How Global-Genome Nucleotide Excision Repair Enters and Exits Chromatin

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Summary

The genome of every cell in every organism serves as the blueprint of cellular function and shape. It's therefore not surprising, that high requirements are posed against the chemical molecule deoxyribonucleic acid (DNA), which contains the whole genetic information such as stability and the possibility to copy the genetic information. Unfortunately, exogenous influences like chemicals, UV light or ionising radiation may harm the DNA. Even endogenous substances like free oxygen radicals negatively interact with the DNA. Unrepaired lesions may be fixed to the DNA which end up in diseases like cancer. Humans as well as many other species have specialized DNA repair mechanism to deal with these kind of threats. The damage is either repaired directly or the progression of the cell cycle is stopped or even a programmed cell death is initiated.

UV light is an invisible radiation emitted from the sun with a wave length between 100 and 380 nanometer. The short-waved majority gets absorbed by the ozone layer and the atmosphere. Therefore, only the so called UV-A and UV-B radiation between 280 and 380 nanometer is physiological relevant. On one hand, UV light is indispensable for the photosynthesis and biological energy production and therefore essential for life on earth, but on the other hand it's a threat for the integrity of the cell. In case UV light hits the DNA of a skin cell two different photochemical reactions may occur: The formation of cyclobutane pyrimidine dimer (CPD) and (6-4)-photoproduct (6-4PP). The inability to repair those UV lesions leads to the devastating disease Xeroderma pigmentosum, which is characterized by pronounced light sensitivity, skin lesions and a high probability for skin cancer.

The specific and evolutionary conserved repair mechanism *nucleotide excision repair (NER)* is able to recognize and repair those lesions. The repair is performed in a three step process: The recognition of the damaged nucleotides, the removal of the damaged fragment, resynthesis of the missing DNA using the complimentary strand. Noteworthy that not the chemical damage itself gets fixed but the damaged fragments are being replaced.

To minimize the space taken by the up to 2 m long DNA double strand helix, the DNA is compacted in the nucleus in form of chromatin. Chromatin is a complex between proteins (histones and other chromatin forming proteins) and DNA. However, the compaction of the

DNA is a burden for the systematic scanning of the DNA for lesions as the DNA accessibility is impaired.

In this work we investigated how the cell is able to recover from UV induced DNA lesions, even though the chromatin is a severe barrier for successful DNA repair. We could show that the repair activity is supported by the protein *chromodomain helicase DNA binding protein 1* (CHD1). CHD1 interacts with the chromatin monomers, the nucleosomes, and removes them from the lesion to allow recruitment of the NER repair proteins. Cells missing CHD1 suffer from reduced NER repair efficiency and decreased long term UV survival. CHD1 can be considered as tumor suppressor as the lack of the protein may trigger cancerogenesis.

Zusammenfassung

Das Erbgut eines jeden Lebewesens dient als Bauplan und Funktionsbestimmung der Zellen und somit des ganzen Organismuses. Es ist daher nicht überraschend, dass an das Molekül Desoxyribonukleinsäure (DNS), welches die Erbinformationen enthält, hohe Ansprüche wie Stabilität und die Möglichkeit die Geninformation zu kopieren, gestellt werden. Bedauerlicherweise wird die DNS aber stetig von äusseren Einflüssen wie chemische Substanzen, UV Licht oder ionisierender Strahlung beschädigt. Auch Stoffe innerhalb der Zellen (z.B. freie Sauerstoffradikale) können das Erbgut chemisch verändern. Die Gefahr besteht, dass die so entstanden DNS-Schäden das Genom des Organismus mutieren und sich so die Zellfunktionen verändern. Mutationen im Erbgut können unter gewissen Umständen zu Krankheiten wie Krebs führen. Menschliche Zellen besitzen spezialisierte Mechanismen um diese Konsequenzen zu verhindern. Dabei wird der Schaden entweder direkt repariert, das Fortschreiten des Zellzyklus gehemmt oder sogar der programmierte Zelltod ausgelöst.

UV-Licht, eine unsichtbare Strahlung im Wellenlängenbereich zwischen 100 und 380 Nanometern, wird von der Sonne ausgestrahlt. Ein kurzwelliger Grossteil davon wird von der Ozonschicht und der Erdatmosphäre wieder absorbiert. Dadurch ist nur der Bereich zwischen 280 und 380 Nanometern, die sogenannten UV-A und UV-B Anteil physiologisch relevant. UV-Licht ist *einerseits* unverzichtbar für die Photosynthese und die biologische Energiegewinnung und somit essentiell für das Leben auf der Erde, *anderseits* schädigt UV-Licht die Integrität der Zelle. Trifft UV-Licht auf die DNS der Hautzellen, bilden sich zwei verschiedene chemische Photoprodukte: *Cyclobutan-Pyrimide-Dimer* (CPD) und *(6-4)-Photoprodukt* ((6-4)PP). Die Unfähigkeit diese Schäden zu reparieren zeigt sich durch die Krankheit Xeroderma Pigmentosum, welche sich durch eine ausgeprägte Lichtsensitivität, Hautläsionen und eine hohe Wahrscheinlichkeit an Hautkrebs zu erkranken auszeichnet.

Der spezifische und evolutiv über viele Spezien konservierte Reparaturmechanismus *Nukleotide-Exzision-Reparatur* ist in der Lage diese Schäden zu erkennen und zu reparieren. Die Reparatur erfolgt in drei Schritten, der Erkennung des Schadens durch die Proteine UV-DDB und XPC, der Entfernung des geschädigten Strangs und der Neusynthese mittels des komplementären DNS-Strangs. Somit wird nicht der chemische Schaden selbst repariert, aber die beschädigten Nukleotide ersetzt.

Um den Platzverbrauch des bis zu zwei Meter langen DNS-Moleküls in Form von Chromosomen möglichst gering zu halten, wird es in Form von Chromatin im Zellkern aufgewickelt. Chromatin ist ein Komplex aus Proteinen (Histone und weitere chromatin-bildende Proteine) und DNS. Das systematische Absuchen der DNS auf Schädigung wird aber durch das Vorhandensein von Chromatin erschwert.

In dieser Arbeit wurde untersucht, wie sich die Zelle von der durch UV-Licht induzierte Schädigung wieder erholen kann, obwohl das Chromatin eine zu überwindende Barriere darstellt. Wir konnten zeigen, dass das Protein Chromodomäne-Helikase DNS-bindendes Protein 1 (CHD1) die Reparaturaktivität unterstützt und fördert. CHD1 interagiert mit den Chromatineinheiten, den Nucleosomen und entfernt sie vom Schaden weg. Die NER-Proteinen können so ungehindert den Reparaturvorgang einleiten. Ohne CHD1 ist die Rekrutierung von Reparaturproteinen gestört, was dazu führt, dass die Reparatureffizienz sinkt und die Überlebensfähigkeit von menschlichen Zellen langfristig beeinträchtigt. CHD1 kann somit als Tumorsuppressor betrachtet werden, da ein Nichtvorhandensein die Tumorbildung unterstützt.

Abbreviations

6-4PP	(6-4) photoproduct
ATP	Adenosine triphosphate
CHD1	Chromo domain helicase DNA binding protein 1
CPD	Cyclobutane pyrimidine dimer
DDB2	DNA damage-binding protein 2
EdU	5-ethynyl-2'-deoxyuridine
ERCC1	Excision repair cross-complementing 1
GG-NER	Global-genome nucleotide excision repair
MNase	Micrococcal nuclease
NER	Nucleotide excision repair
RAD23B	Human homolog of RAD23, B
TC-NER	Transcription coupled nucleotide excision repair
TFIIH	Transcription factor IIH
UV	Ultraviolet
XPA-XPG	Xeroderma pigmentosum group A-G complementing group protein

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1 Introduction

1.1 Regulation of Global-Genome Nucleotide Excision Repair

Section 1.1 constitute an article (Rüthemann *et al*, 2016) published 2016 in the journal *Frontiers in Genetics* and is entitled:

Global-genome Nucleotide Excision Repair Controlled by Ubiquitin/Sumo Modifiers

This review explains nucleotide excision repair and its regulation by ubiquitin and sumo modifiers. I prepared Figure 1 and collaborated with *C. Balbo Pogliano for* Figure 2 and 3. The manuscript has been written together with *Hanspeter Nägeli.*

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REVIEW
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Global-genome Nucleotide Excision Repair Controlled by Ubiquitin/Sumo Modifiers

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Global-genome nucleotide excision repair (GG-NER) prevents genome instability by excising a wide range of different DNA base adducts and crosslinks induced by chemical carcinogens, ultraviolet (UV) light or intracellular side products of metabolism. As a versatile damage sensor, xeroderma pigmentosum group C (XPC) protein initiates this generic defense reaction by locating the damage and recruiting the subunits of a large lesion demarcation complex that, in turn, triggers the excision of aberrant DNA by endonucleases. In the very special case of a DNA repair response to UV radiation, the function of this XPC initiator is tightly controlled by the dual action of cullin-type CRL4^{DDB2} and sumo-targeted RNF111 ubiquitin ligases. This twofold protein ubiquitination system promotes GG-NER reactions by spatially and temporally regulating the interaction of XPC protein with damaged DNA across the nucleosome landscape of chromatin. In the absence of either CRL4^{DDB2} or RNF111, the DNA excision repair of UV lesions is inefficient, indicating that these two ubiquitin ligases play a critical role in mitigating the adverse biological effects of UV light in the exposed skin.

Keywords: aging, cyclobutane pyrimidine dimer, DNA repair, genomic instability, photoproducts, sunburns, skin cancer, UV radiation

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INTRODUCTION

All organisms are constantly under attack by environmental and endogenous DNA-damaging agents that endanger the sequence fidelity of their genomes. Many environmental mutagens cause "bulky" DNA adducts that destabilize the complementary pairing of bases in the native double helix (Straub et al., 1977; Knox et al., 1987). Base pair-destabilizing lesions also result from internal by-products of cellular metabolism including oxygen radicals (Brooks et al., 2000;

Abbreviations: 6-4PP, (6-4) pyrimidine–pyrimidone photoproduct; BHD, β-Hairpin domain; CETN2, centrin 2; CPD, cyclobutane pyrimidine dimer; CUL4A, cullin 4A; DDB, damaged DNA-binding; ERCC1, excision repair cross-complementing 1; GG-NER, global-genome nucleotide excision repair; MPG, methylpurine-DNA glycosylase; NER, nucleotide excision repair; NEDD8, neural precursor cell expressed developmentally down-regulated 8; Npl4, nuclear protein localization 4 homolog; Oct4, octamer binding transcription factor 4; OGG1, 8-Oxo-guanine-DNA glycosylase; OTUD4, OTU deubiquitinase 4; RAD23B, human homolog of RAD23, B; RNF111, RING finger protein 111; RPA, replication protein A; ROC1 regulator of cullins 1; RPS27A, ubiquitin-40S ribosomal protein S27A; SMUG1, single strand-selective monofunctional uracil-DNA glycosylase 1; Sox2, sex determining region Y (SRY)-box 2; Sumo, small ubiquitin-related modifier; TC-NER, transcription-coupled nucleotide excision repair; TDG, thymine-DNA glycosylase; TFIIH, transcription factor IIH; TG, transglutaminase-like; UBA52, ubiquitin A-52; UBB, ubiquitin-B; UBC, ubiquitin-C; USP7, ubiquitin-specific processing protease 7; Ufd1, ubiquitin fusion degradation 1; UV, ultraviolet; VCP, valosin-containing protein; XP, xeroderma pigmentosum.

Kuraoka et al., 2000), but the most common type of bulky DNA lesion arises from the UV spectrum of sunlight or indoor tanning devices, generating covalent crosslinks joining neighboring pyrimidines, i.e., CPDs and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs; Brash, 1988). If not readily repaired, these pyrimidine crosslinks and other bulky adducts interfere with transcription, DNA replication or cell cycle progression (Lopes et al., 2006; Brueckner et al., 2007), eventually giving rise to mutations and chromosomal aberrations that accelerate aging and culminate in cancer (Marteijn et al., 2014). Unfortunately, the incidence of skin cancer continues to increase and remains a public health concern despite widespread knowledge that excessive exposure to sunlight is the major risk factor for cutaneous neoplasms (Donaldson and Coldiron, 2011; Usher-Smith et al., 2014). This review is focused on recent advances in our knowledge of how polypeptide modifiers regulate the DNA repair response preventing sunlight-induced skin cancer.

Excision of Bulky DNA Lesions

Nucleotide excision repair is a molecular cut-and-patch machine that removes bulky base lesions by incising damaged DNA strands on either side of the injury, thereby eliminating 24- to 32-nucleotide long single-stranded segments (Huang et al., 1992; Moggs et al., 1996). Depending on their location in the genome, bulky lesions are sensed by two alternative mechanisms. The TC-NER pathway is initiated when an RNA polymerase II complex encounters obstructing base lesions (Bohr et al., 1985). Such transcriptional roadblocks trigger a stepwise reaction for the rapid removal of base lesions from transcribed strands (reviewed by Hanawalt and Spivak, 2008; Vermeulen and Fousteri, 2013; Marteijn et al., 2014). On the other hand, GG-NER activity is generally slower but detects bulky lesions anywhere in the genome independently of transcription (reviewed by Scharer, 2013; Puumalainen et al., 2016). Genetic defects in the GG-NER pathway cause XP, which is a severe cancer-prone syndrome presenting with photosensitivity, extreme sunburns and an over 1,000-fold higher risk of contracting sunlight-induced neoplasms of the skin (Hollander et al., 2005; DiGiovanna and Kraemer, 2012). Patients suffering from the XP syndrome are classified into distinct genetic complementation groups (from XP-A to XP-G) reflecting mutations in respective NER genes (Cleaver et al., 2009). A variant form of this disease (XP-V) is caused by mutations in a gene coding for DNA polymerase η that catalyzes with high nucleotide sequence fidelity the replicative bypass of UV lesions in S phase of the cell division cycle (Masutani et al., 1999).

The initial detection of bulky lesions in the GG-NER pathway is carried out by a three-subunit factor consisting of XP group C protein (XPC; Sugasawa et al., 1998; Volker et al., 2001) one of two human RAD23 homologs (predominantly RAD23B; Ng et al., 2003) and (CETN2, (Araki et al., 2001; Nishi et al., 2005; Dantas et al., 2011). The DNA-binding activity of this heterotrimeric complex resides with the XPC subunit itself. RAD23B and CETN2 contribute by supporting the proper folding of XPC protein and by protecting this DNA-binding subunit from degradation (Ng et al., 2003; Xie et al., 2004; Krasikova et al., 2012). Although RAD23B stimulates the recognition of damaged DNA by XPC protein (Sugasawa et al., 1996), it is readily released once XPC associates with DNA lesion sites (Fei et al., 2011; Bergink et al., 2012). Conversely, CETN2 remains associated with target sites (Dantas et al., 2013) where XPC provides a platform for the recruitment of TFIIH. This 10-subunit complex contains an ATPase (XPB) and a DNA helicase (XPD) that separate complementary strands to produce an unwound configuration of about 25 nucleotides around the lesion (Evans et al., 1997; Wakasugi and Sancar, 1998). Stability to the resulting open intermediate or "bubble" is conferred by XPA together with RPA, until the DNA strand containing the damage is incised by structure-specific endonucleases exactly at the double-stranded to single-stranded DNA transitions on each side of the bubble (Evans et al., 1997; Missura et al., 2001; Li et al., 2015). A protein heterodimer composed of XPF and ERCC1 introduces the incision on the 5′ side, followed by incision on the 3′ side by the endonuclease activity of XPG (Staresincic et al., 2009). After this dual incision and consequent release of the excised oligonucleotide carrying the damage, the remaining single-stranded gap is filled by DNA repair synthesis by the action of DNA polymerases η, ε, or κ (Ogi et al., 2010). Ligation by DNA ligase I and DNA ligase IIIα finally restores helix integrity (Araujo et al., 2000; Moser et al., 2007).

Structure and Interactome of the XPC Initiator

The human XPC polypeptide is made of 940 amino acids and harbors domains for binding to DNA (Hey et al., 2002; Yasuda et al., 2005; Trego and Turchi, 2006) and multiple protein partners (**Figure 1**). Its molecular structure can be extrapolated from that of Rad4 protein, the evolutionarily conserved homolog in the yeast *Saccharomyces cerevisiae* (Min and Pavletich, 2007). When undergoing co-crystals with a model bulky lesion in duplex DNA, Rad4 protein deploys four adjacent domains for substrate binding by two different modalities. One part makes use of a TG domain and a BHD1, which cooperate in associating with 11 base pairs of duplex DNA flanking the damaged site. The second part uses two further BHD2 and BHD3 to interact with four consecutive nucleotides of the undamaged DNA strand opposing the flipped-out bulky lesion. No interactions at all are formed with the lesion itself. In human XPC protein, this β-hairpin region (BHD1–3) interacting indirectly with damaged sites encompasses amino acids 637–831 (Camenisch et al., 2009).

In addition to mediating associations with substrate DNA, the TG domain is required for the interaction between Rad4 and Rad23 (Min and Pavletich, 2007), and between the corresponding human homologs XPC and RAD23B. A fraction of the human TG domain also interacts with XPA protein (Bunick et al., 2006). Another partner, known as DDB2 does not exist in lower eukaryotes like yeast. However, a transient association between DDB2 and XPC is critical for the processing of CPDs in mammals (Itoh et al., 2004) and the respective contact

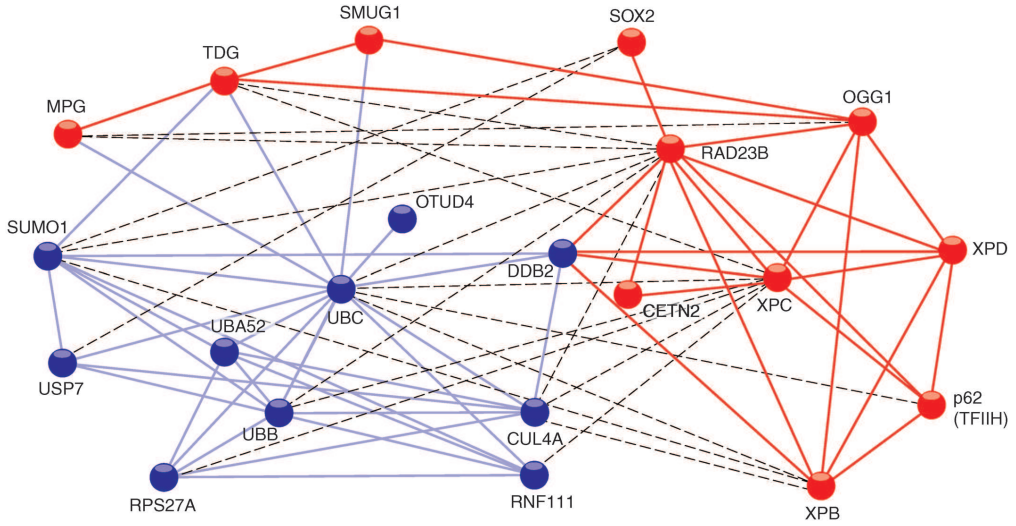

FIGURE 1 | STRING network view of XPC interactions with proteins. The connecting lines indicate proven or predicted interactions using the http://www.string-db.org information source. The different colors of the protein nodes reflect their clustering in two groups according to the KMEANS algorithm (Brohée and Helden, 2006). Blue nodes, ubiquitin-related proteins; red nodes, DNA repair proteins. Blue lines, interactions between ubiquitin-related proteins; red lines, interactions between DNA repair proteins. The dashed lines highlight interactions between the two different clusters.

sites have been mapped to the TG and BHD1 regions (Fei et al., 2011). Residues 847–863 in the carboxy-terminus of human XPC form an α-helix that binds tightly to CETN2 (Nishi et al., 2005; Yang et al., 2006). Amino acid residues 816–940 located in this carboxy-terminus and a portion of the amino-terminal region around amino acid position 334 make contacts with two members (p62 and XPB) of the 10-subunit TFIIH complex (Yokoi et al., 2000; Uchida et al., 2002; Bernardes de Jesus et al., 2008). These particular interactions reflect the actual role of XPC in recruiting the XPD helicase, another TFIIH subunit, which in turn detects lesions by scanning DNA and sequestering damaged nucleotides in a dedicated recognition pocket on its enzyme surface (Sugasawa et al., 2009; Mathieu et al., 2010). In addition, XPC protein interacts with the following base excision repair enzymes: MPG, (Miao et al., 2000), TDG, (Shimizu et al., 2003), OGG1, (D'Errico et al., 2007; Melis et al., 2011), and SMUG1, (Shimizu et al., 2010). This crosstalk with multiple DNA glycosylases indicates that XPC may adopt a more general function in recruiting diverse repair enzymes to base pair-disrupted sites in the double helix. Perhaps the most unexpected interaction of XPC protein is with the Oct4-Sox2 transcriptional activator. Indeed, the XPC complex was found to serve as a coactivator of the Oct4-Sox2-dependent expression of the Nanog pluripotency gene (Fong et al., 2011; Cattoglio et al., 2015; Zhang et al., 2015). A two-hybrid screen, which used XPC protein as the bait, revealed many further potential interaction partners involved in DNA synthesis, transcription, post-translational modification, proteolysis, signal transduction, and metabolism (Lubin et al., 2014). To date, the biological consequence of these putative associations is unknown. Finally, there are also proven interactions of XPC protein with two different deubiquitinases, i.e., OTUD4, (Lubin et al., 2014) and USP7 deubiquitinase (for *U*biquitin-*S*pecific-processing *P*rotease 7; He et al., 2014). It appears, therefore, that XPC upon ubiquitination becomes a substrate for these two deubiquitinating enzymes.

Support for the XPC Initiator from a Specialized UV Lesion Detector

Exposure of DNA to UV light results in the formation of CPDs and 6-4PPs in a stoichiometry of approximately 3:1. These two kinds of pyrimidine crosslinks differ in their biophysical properties, genomic distribution, and biological effects. First, CPD sites are characterized by a relatively minor destabilization of base pairs compared to duplex DNA containing 6-4PPs (Kim et al., 1995; Jing et al., 1998; McAteer et al., 1998). Second, CPDs are evenly distributed across the chromatin landscape, whereas 6-4PPs are formed preferentially in linker DNA segments rather than in nucleosome cores (Gale et al., 1987; Gale and Smerdon, 1990; Mitchell et al., 1990). Third, because CPDs are removed at slower rates than 6-4PPs, they display a higher mutagenic potential and are responsible for most adverse short- and long-term effects of UV radiation such as sunburns, skin aging and cutaneous cancer (Schul et al., 2002; Garinis et al., 2005).

Despite being the generic repair initiator for all bulky lesions including the slowly repaired CPDs, XPC protein does not bind CPDs in duplex DNA with any appreciable selectivity (Sugasawa et al., 2001; Hey et al., 2002; Reardon and Sancar, 2003; Wittschieben et al., 2005). This lack of specificity for CPDs is, however, compensated by DDB2 protein, which is the factor mutated in XP-E patients (Nichols et al., 2000; Kulaksiz et al., 2005). Unlike XPC, which functions as a non-specific sensor of helix-disrupting bulky lesions, DDB2 is exclusively dedicated to the detection of CPDs and 6-4PPs (Tang et al., 2000). Structural analyses of DDB2 crystals revealed a recognition hole in its

central β-propeller fold that only accommodates CPDs and 6-4PPs while excluding larger base adducts (Scrima et al., 2008; Fischer et al., 2011; Yeh et al., 2012; Osakabe et al., 2015). Notably, the complete lack of functional DDB2 protein in XP-E patients abolishes the repair of CPDs but the excision of 6-4PPs is only marginally affected (Hwang et al., 1999; Moser et al., 2005).

A generally proposed model is that DDB2 recognizes CPDs and, thereafter, delivers them to the XPC partner for initiation and execution of the GG-NER process (Tang et al., 2000; Wakasugi et al., 2001; Fitch et al., 2003). It has been demonstrated that XPC lends two of its previously mentioned DNA-binding folds (TG domain and BHD1) to interact in a transient manner with DDB2 associating with UV lesions. This dynamic DDB2-XPC-DNA intermediate at the damage site allows for the insertion, into the DNA double helix, of a β-hairpin extension protruding from BHD3, eventually competing DDB2 away from the damage (Fei et al., 2011; Mu et al., 2015). Thermodynamically, this β-hairpin insertion by XPC takes place at a considerable energetic cost for local breakage of stacking and hydrogen bond interactions between the involved bases (Mu et al., 2015). The 6-4PPs, being more base pair-disruptive, facilitate this β-hairpin insertion by reducing the helical stability at damaged sites, but XPC protein depends on DDB2 to interact in a productive manner with CPD sites. Thus, the different degree of local helical distortion explains the specific defect of XP-E cells in eliminating CPD lesions.

Polypeptide Modifiers Targeting XPC Protein

In view of the manifold implications of XPC as a generic DNA quality sensor in GG-NER that, in addition, associates with several DNA glycosylases and is responsible for non-repair functions in transcription (see above), it is not astonishing to observe that the activity, cellular level and localization of XPC protein is tightly controlled. For example, it has become clear that various polypeptide modifiers regulate the action of this versatile repair initiator during the cellular response to UV damage.

In addition to its role as a specific UV lesion detector, the DDB2 subunit cooperates with the adaptor DDB1 to recruit the CUL4A scaffold and the RING finger protein ROC1, which together build the $CRL4^{DDB2}$ ubiquitin ligase. By mediating the covalent attachment of one or more 8-kDa ubiquitin moieties to target proteins (Groisman et al., 2003), this cullin-type ligase is able to fine-tune GG-NER activity. Under steady-state conditions, the $CRL4^{DDB2}$ ubiquitin ligase is kept in an inactive form thanks to an association with the COP9 signalosome, a multi-subunit regulatory protease (Fischer et al., 2011). Following the detection of UV lesions by DDB2, COP9 is released giving way to a covalent modification of CUL4A with the ubiquitin-like polypeptide NEDD8, thus activating the ubiquitin ligase complex that, in turn modifies nearby located substrates with Lys48-linked ubiquitin chains (Scrima et al., 2008). The principal ubiquitination substrates include histones H2A, H3 and H4 as well as DDB2 itself and its DNA recognition partner XPC (Nag et al., 2001; Sugasawa et al., 2005; Kapetanaki et al., 2006; Wang et al., 2006; Guerrero-Santoro et al., 2008).

It has been proposed that the $CRL4^{DDB2}$-mediated ubiquitination of histones in response to UV radiation helps opening chromatin, thus facilitating access of the GG-NER repair machinery to damaged DNA (Wang et al., 2006). However, this view is contradicted by the finding that CUL4A conditional-knockout mice show more proficient rather than reduced GG-NER activity (Liu et al., 2009). There is, on the other hand, general agreement that the self-ubiquitination of DDB2 not only suppresses its binding to DNA but also promotes its degradation by the 26S proteasome (Sugasawa et al., 2005). The same $CRL4^{DDB2}$ ligase also ubiquitinates XPC but, unlike the fate of DDB2, XPC retains its DNA-binding property and is shielded from proteasomal breakdown (Sugasawa et al., 2005; Matsumoto et al., 2015). In addition, the XPC protein is modified with Lys63-linked ubiquitin chains by another ligase complex referred to as RNF111 or Arkadia (Poulsen et al., 2013). This extra ubiquitination reaction is strictly dependent on the prior UV-dependent modification of XPC protein with sumo, defining RNF111 as a sumo-targeted ubiquitin ligase (Wang et al., 2005).

In summary, GG-NER activity upon UV damage is coordinated by several polypeptide modifiers including NEDD8, sumo, Lys48- and Lys63-linked ubiquitin chains. Sumo and the two aforementioned ubiquitin chains decorate XPC protein at multiple covalent modification sites. Interestingly, *in situ* immunofluorescence studies indicate that a down-regulation of $CRL4^{DDB2}$ or RNF111 activity has opposite effects by inhibiting and stimulating, respectively, the accumulation of XPC in damage spots generated by UV irradiation through micropore filters. This observation raises the possibility that Lys48-linked ubiquitin chains (produced by $CRL4^{DDB2}$) and Lys63-linked counterparts (produced by RNF111) have distinct modulating roles. The function of Lys48-linked ubiquitin chains in regulating XPC is discussed in the next section below. With regard to the accompanying sumo modification, this reaction has been implicated in promoting the release of DDB2 once XPC is bound to UV lesion sites. In the absence of XPC sumoylation, both DDB2 and XPC are trapped together on damaged DNA carrying the lesion, thus posing a block to downstream NER steps (Akita et al., 2015). Since RNF111 is targeted to protein substrates by sumo residues, it is tempting to propose that the effect of sumoylation in releasing XPC may actually be executed by a subsequent attachment of Lys63-linked ubiquitin chains by RNF111. This functional link between sumo and Lys63-linked ubiquitin would explain the persistence of XPC in UV lesion spots observed by Poulsen et al. (2013) and van Cuijk et al. (2015) following RNF111 depletion.

Dynamic Relocation of XPC in Damaged Chromatin

The genome packaging in eukaryotic cells is imposed by two very diverging needs. The DNA filaments must be compressed to fit into the narrow cellular nucleus but nevertheless remain accessible to the diverse nuclear transactions. To achieve this double requirement, DNA is assembled with histones to

generate a tight but dynamic array whose repeating unit is the nucleosome (reviewed by Khorasanizadeh, 2004; Thoma, 2005). Each individual nucleosome displays a core particle, where 147 base pairs of duplex DNA are wrapped around a core histone octamer (two each of H2A, H2B, H3, and H4) and a DNA spacer or "linker" of variable length. Also, in higher eukaryotes histone H1 associates with linker DNA segments to induce further packaging allowing for increased compaction of the DNA double helix.

It is of paramount importance to address the possible regulatory role of polypeptide modifiers in the GG-NER pathway taking into account this chromatin context. New insights into the function of $CRL4^{DDB2}$-mediated ubiquitination came from the enzymatic partitioning of chromatin by incubation with micrococcal nuclease (MNase). This particular enzyme breaks down DNA in the more accessible linker segments much faster than in the less accessible nucleosome cores. As a consequence, the incubation of chromatin with MNase produces a soluble supernatant of mostly non-histone proteins that, before MNase digestion, were associated with linker DNA segments spacing the nucleosomal core particles (amounting to ∼35% of total genomic DNA). Even when saturating enzyme concentrations are used, however, MNase digestions of chromatin leave behind the vast majority of nucleosome core particles (amounting to ∼60% of total DNA) in the form of an insoluble nucleoprotein fraction (Telford and Stewart, 1989). Two previous findings led us to predict that, in response to UV irradiation, $CRL4^{DDB2}$ activity would not be uniformly distributed along nucleosome arrays. First, DDB2 protein, the DNA-binding subunit of $CRL4^{DDB2}$, associates with > 10-fold higher affinity with 6-4PPs ($K_a = 1.5 \times 10^9$ M^{-1}) relative to CPDs ($K_a = 1 \times 10^8$ M^{-1}; (Reardon et al., 1993; Wittschieben et al., 2005). Second, 6-4PPs are formed mainly in internucleosomal linker DNA (Gale and Smerdon, 1990; Mitchell et al., 1990). Therefore, we were not surprised to find that DDB2 associates preferentially, although not exclusively, with 6-4PPs situated in accessible MNase-sensitive internucleosomal segments (Fei et al., 2011). Coversely, it was believed that XPC is unable to interact with DNA assembled with histone octamers forming nucleosome cores (Yasuda et al., 2005) but, against this prevailing notion, MNase digestions of chromatin revealed that XPC protein associates rather evenly with nucleosome core particles and internucleosomal linker segments. Upon UV irradiation, this interaction of XPC protein with nucleosome core particles is stimulated (Fei et al., 2011). This latter finding is in line with structural analyses of core particle crystals containing a site-directed UV damage, which revealed that the tight wrapping around histone octamers increases the DNA flexibility at lesion sites (Osakabe et al., 2015). This higher flexibility may, in turn, explain how XPC protein is able to carry out, even in the nucleosome core context, its indirect damage sensor function by binding to the undamaged strand opposing bulky lesions.

In agreement with the selectivity of the DDB2 subunit for UV lesions in internucleosomal linker DNA, following UV radiation the whole $CRL4^{DDB2}$ ubiquitin ligase is relocated mainly to these highly amenable sites. Due to this distinctive positioning of $CRL4^{DDB2}$, the modification with Lys48-linked ubiquitin chain takes place more efficiently on XPC bound to internucleosomal DNA, whereas XPC molecules on core particles are less prone to ubiquitination (Fei et al., 2011). The role of $CRL4^{DDB2}$ in this context was confirmed by the following experimental manipulations: (i) depletion of either DDB2 or CUL4A using RNA interference, (ii) depletion of the nuclear ubiquitin pool by using the proteasome inhibitor MG132, or (iii) suppression of the ubiquitin pathway using a small-molecule E1 inhibitor. Alternatively, the ubiquitination of XPC was inhibited in mouse cells expressing a temperature-sensitive E1 mutant or with an XPC-green fluorescent fusion protein that makes the XPC protein refractory to ubiquitination. After each of these experimental manipulations, the XPC molecules were devoid of ubiquitin moieties and, as a consequence, almost completely relocated to nucleosome core particles (Fei et al., 2011). These findings demonstrate that one of the functions of $CRL4^{DDB2}$-mediated ubiquitination is to retain XPC molecules at internucleosomal sites, which constitute DNA repair hotspots for the effective recruitment of TFIIH and further downstream NER factors (**Figure 2**). In the absence of $CRL4^{DDB2}$ activity, more XPC binds to CPDs located in nucleosome core particles representing a less permissive chromatin environment with poor recruitment of downstream GG-NER factors. We concluded that the $CRL4^{DDB2}$-mediated ubiquitination serves to establish a distinctive spatiotemporal distribution of the XPC sensor during the UV damage response, in particular to optimize the recruitment of NER factors in mammalian chromatin.

Ubiquitin-dependent Extraction of DDB2 and XPC from Chromatin

Although the DDB2 damage detector is required for efficient recognition and excision of CPDs, Lys48-linked ubiquitin moieties elicit its proteolytic breakdown within few hours after exposure to UV light (Nag et al., 2001; Rapic-Otrin et al., 2002). This precipitous self-ubiquitination and degradation of DDB2 provides a time switch that limits the $CRL4^{DDB2}$ ubiquitin ligase activity, and its regulatory effect on the XPC partner, to a short period after acute UV pulses. Due to DDB2 degradation, the proportion of ubiquitinated XPC diminishes progressively and, therefore, XPC can relocate from internucleosomal DNA segments to not yet processed residual UV lesions, essentially CPDs, located within the less amenable nucleosome core particles (Fei et al., 2011). These dynamic chromatin transitions, involving degradation of DDB2 and relocation of XPC, are triggered by the ubiquitin-selective p97 segregase, also known as VCP, (Puumalainen et al., 2014). Hexameric assemblies of p97 subunits convert ATP hydrolysis into mechanical activity to liberate ubiquitinated proteins from diverse subcellular substrates (Rouiller et al., 2000; Zhang et al., 2000). That p97 hexamers recognize ubiquitinated DDB2 and XPC was first demonstrated *in situ* on UV lesions spots in the nuclei of human cells. Second, it was confirmed biochemically that Lys48-ubiquitinated DDB2, XPC, and p97 are found in the same multi-protein complex (Puumalainen et al., 2014). This p97 recruitment to ubiquitinated DDB2 and XPC depends

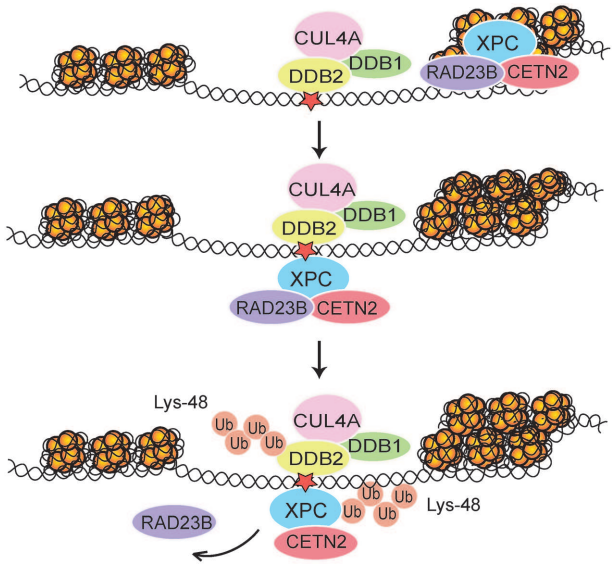

FIGURE 2 | Regulation of XPC localization in chromatin. After each UV pulse, the cullin-type CRL4^{DDB2} ligase complex (comprising *inter alia* DDB1, DDB2, and CUL4A) is recruited mostly to accessible internucleosomal sites in chromatin. The ensuing modification of XPC with Lys48-linked ubiquitin (Ub) chains leads to a temporary retention of XPC on internucleosomal DNA, thus reducing its constitutive association with nucleosome core particles (Fei et al., 2011). Subsequently, RAD23B is released and the XPC-CETN2 heterodimer provides a platform for recruitment of the TFIIH complex. The UV radiation damage is symbolized by a red star.

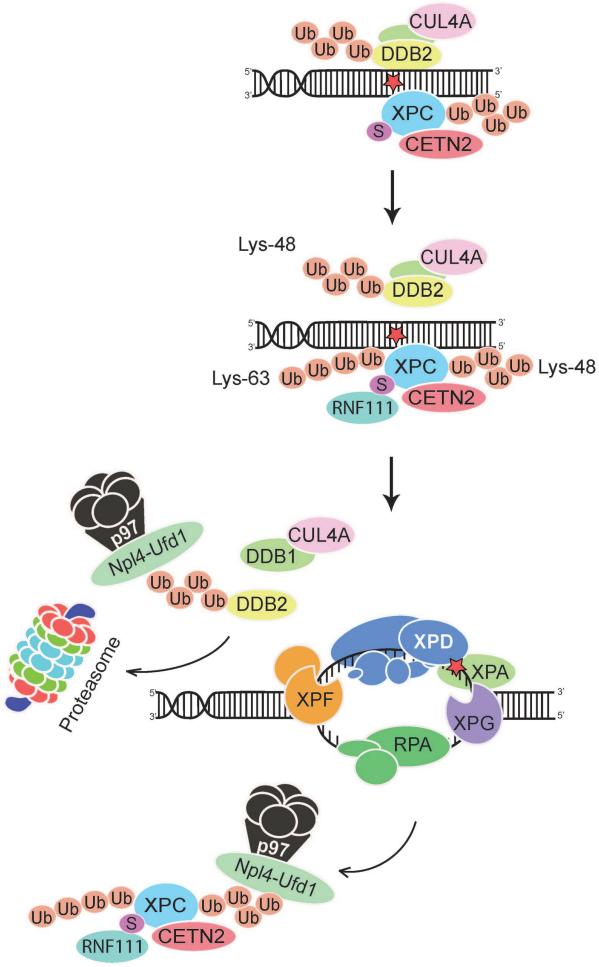

FIGURE 3 | Extraction of DDB2 and XPC from chromatin. The p97 segregase coordinates GG-NER activity by removing Lys48-ubiquitinated DDB2 and Lys48-ubiquitinated XPC from chromatin, thus promoting downstream recognition (by the XPD subunit of TFIIH in conjunction with XPA and RPA) and double DNA incision. The XPC subunit is thought to leave the preincision complex after recruitment of TFIIH but before engagement of the DNA endonucleases XPF-ERCC1 and XPG (Scharer, 2013; van Cuijk et al., 2015). Ubiquitinated DDB2 is forwarded to the proteasome for degradation, whereas XPC is recycled by de-ubiquitination (He et al., 2014; Lubin et al., 2014; Puumalainen et al., 2014). Lys63-linked ubiquitin chains on XPC may further enhance these dynamic relocations at UV lesions by favoring the dissociation of DDB2 from XPC. See text for further details on the postulated dual role of CRL4^{DDB2} (generating Lys48-linked ubiquitin chains) and RNF111 (generating Lys63-linked ubiquitin chains) in regulating GG-NER activity. Npl4-Ufd1, adaptor complex that confers specificity to the p97 segregase; the UV radiation damage is symbolized by a red star.

on adapter proteins (Meyer et al., 2000; Hänzelmann et al., 2011) known to confer substrate specificity to the p97 segregase (**Figure 3**).

Next, the p97 function was down regulated by RNA interference or, alternatively, by expression of a dominant-negative mutant (Ye et al., 2003) that still displays substrate-binding but is unable to exert segregase activity and, therefore, remains trapped on ubiquitinated proteins. The consequence of this diminished p97 activity is an enrichment of DDB2 and XPC in UV lesion spots, thus reflecting an excessive accumulation of these factors in damaged chromatin. The down-regulation of p97 inhibited the UV-induced proteolytic clearance of DDB2 and also increased the level of ubiquitinated XPC. However, despite their roles in the initiation of GG-NER activity, this induced persistence of DDB2 and XPC impaired UV lesion excision. Moreover, the compromised DNA repair efficiency resulting from p97 down regulation caused hypersensitivity to UV light and enhanced chromosomal aberrations after UV exposure.

The genome instability observed in UV-irradiated cells after p97 depletion was reversed by concurrent down-regulation of DDB2 or XPC (Puumalainen et al., 2014). These findings suggested that the uncontrolled accumulation of DDB2 or XPC is detrimental and that a tight regulation of their levels in chromatin is essential for genome stability. Elaborating on this hypothesis, one would expect that an excessive presence of one of these factors should be sufficient to destabilize the genome. In support of this hypothesis, it was found that under conditions of normal p97 activity, overexpression of wild-type DDB2 but not overexpression of a DNA-binding mutant, compromised UV lesion excision and increased the frequency of chromosomal aberrations following UV irradiation. Importantly, double overexpression experiments generating abnormally high levels of both DDB2 and p97 confirmed the expectation that the negative effects of DDB2 overexpression are reversed by concomitantly increasing p97 levels. Thus, a surplus of DDB2

enhances chromosomal aberrations only as long as its chromatin level exceeds the turnover capacity of the p97 segregase. Taken together, these findings point out that a strict spatial and temporal regulation of the chromatin homeostasis of DDB2 and its XPC partner by the p97 segregase is crucial for GG-NER activity (**Figure 3**).

CONCLUSION

The XPC complex provides the generic initiator of GG-NER activity on the basis of its ability to sense the damage-dependent disruption of base pairs in double-stranded DNA and recruit the XPD scanner for bulky lesion recognition. An intriguing peculiarity of the XPC complex is that its function in initiating the excision of UV lesions is tightly regulated by NEDD8, sumo and ubiquitin modifiers. This special regulation is apparently not needed for the recognition and excision of other bulky lesions induced by chemical carcinogens or endogenous metabolic byproducts. An evolutionary perspective may help to understand the unique need for polypeptide modifier-dependent regulation of GG-NER activity in response to UV irradiation.

Evolution of life on our planet would have failed without the emergence of an effective DNA repair function dealing with UV lesions. Indeed, a vast majority of living organisms exposed to sunlight display rapid, efficient and secure molecular tools for the repair of UV lesions consisting of DNA photolyases. By visible light-driven catalysis, these DNA photolyases revert pyrimidine dimers (CPDs and 6-4PPs) to pyrimidine monomers without excision of bases, nucleoside or nucleotide residues (Sancar, 2003; Weber, 2005). Unlike other animals, however, placental mammals are devoid of this light-dependent DNA repair reaction, possibly because they originated from nocturnal ancestors (Essen and Klar, 2006). While returning to a diurnal life under sunlight, placental mammals were left with the GG-NER pathway (also known as "dark repair") as the only means to process UV lesions in the exposed skin. In principle, many potential problems arise with this upgrade of GG-NER activity as the unique DNA repair defense against UV lesions. First, CPDs would escape repair because the XPC initiator is not able to detect this prevalent type of UV lesion. Second, once exposed to sunlight, skin cells would be faced with the simultaneous and uncontrolled cleavage of their genomic DNA at thousands or more chromosomal sites, which constitutes a striking threat to genome stability. Third, CPDs are formed evenly across the genomic DNA, including compacted chromatin sites that are poorly amenable to the GG-NER machinery.

The present review highlights NEDD8-, sumo- and ubiquitin-dependent mechanisms by which these problems related to "dark repair" by the GG-NER machinery are mitigated in human skin cells. First, the dedicated UV damage sensor DDB2 recruits its XPC partner to CPD lesions that, without DDB2, would remain undetected. Second, the GG-NER-initiating activity of XPC undergoes a tight spatial regulation. By recruitment of the $CRL4^{DDB2}$ ligase responsible for XPC ubiquitination, the GG-NER reaction is in the beginning directed to highly amenable internucleosomal DNA segments that are accessible to downstream excision factors, thus protecting more compacted chromatin sites from premature incisions that might favor the fragmentation of chromosomes. Third, the repair-initiating activity of XPC undergoes a tight temporal regulation. By means of proteolytic breakdown triggered by the $CRL4^{DDB2}$ ubiquitin ligase, the repair-stimulating action of DDB2 is self-limiting after an acute pulse of UV damage. Fourth, the physical interaction between DDB2 and XPC is counter-regulated by sumo and, presumably, the sumo-dependent RNF111 ubiquitin ligase. It is still an enigma how DDB2 and the XPC complex take advantage of histone-modifying enzymes as well as chromatin remodelers to relax chromatin regions and initiate the repair of compacted DNA substrates in a coordinated manner. It has, however, become clear that p97-mediated extraction of a surplus of ubiquitinated DDB2 and XPC is necessary to achieve optimal GG-NER activity and avoid molecular collisions with concomitant nuclear processes like transcription or DNA replication. Through addition of these NEDD8-, sumo- and ubiquitin-dependent control circuits, it has become possible during mammalian evolution to upgrade the GG-NER system as the only available DNA repair reaction protecting from UV-induced skin mutagenesis and carcinogenesis.

AUTHOR CONTRIBUTIONS

PR, CBP, and HN wrote the manuscript. PR and CP prepared the figures.

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1.2 Mechanism of Xeroderma Pigmentosum Group C Protein

Section 1.2 constitute an article (Puumalainen *et al*, 2015) published 2015 in the journal *Cellular and Molecular Life Sciences* and is entitled:

Xeroderma pigmentosum group C sensor: unprecedented recognition strategy and tight spatiotemporal regulation

This review explains the diverse roles of XPC in nucleotide excision repair and other cellular functions. The manuscript has been written together with *M. Puumalainen*, *JH. Hyun Min and H. Nägeli.*

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Cellular and Molecular Life Sciences

REVIEW

Xeroderma pigmentosum group C sensor: unprecedented recognition strategy and tight spatiotemporal regulation

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Abstract The cellular defense system known as global-genome nucleotide excision repair (GG-NER) safeguards genome stability by eliminating a plethora of structurally unrelated DNA adducts inflicted by chemical carcinogens, ultraviolet (UV) radiation or endogenous metabolic by-products. Xeroderma pigmentosum group C (XPC) protein provides the promiscuous damage sensor that initiates this versatile NER reaction through the sequential recruitment of DNA helicases and endonucleases, which in turn recognize and excise insulting base adducts. As a DNA damage sensor, XPC protein is very unique in that it (a) displays an extremely wide substrate range, (b) localizes DNA lesions by an entirely indirect readout strategy, (c) recruits not only NER factors but also multiple repair players, (d) interacts avidly with undamaged DNA, (e) also interrogates nucleosome-wrapped DNA irrespective of chromatin compaction and (f) additionally functions beyond repair as a co-activator of RNA polymerase II-mediated transcription. Many recent reports highlighted the complexity of a post-translational circuit that uses polypeptide modifiers to regulate the spatiotemporal activity of this multiuse sensor during the UV damage response in human skin. A newly emerging concept is that stringent regulation of the diverse XPC functions is needed to prioritize DNA repair while avoiding the futile processing of undamaged genes or silent genomic sequences.

Keywords Aging · Diurnal life · DNA repair · Genomic instability · Skin cancer · SUMO · Sunburn · Tumor suppressor · Ubiquitin

Abbreviations

6-4PP	(6-4) Photoproduct
ALC1	Amplified in liver cancer 1
ARF	Alternative reading frame
BER	Base excision repair
BHD	β-Hairpin domain
BRCA1/2	Breast cancer 1/2
CETN2	Centrin 2
CPD	Cyclobutane pyrimidine dimer
CUL4A	Cullin 4A
DDB	Damaged DNA-binding
ERCC1	Excision repair cross-complementing 1
GG-NER	Global-genome nucleotide excision repair
MPG	Methylpurine-DNA glycosylase
NER	Nucleotide excision repair
OGG1	8-Oxo-guanine-DNA glycosylase
OTUD4	OTU deubiquitinase 4
PAR	Poly(ADP-ribose)
PARP1	Poly(ADP-ribose) polymerase 1
RAD23B	Human homolog of RAD23, B
RPA	Replication protein A
ROC1	Regulator of cullins 1

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SIRT1	Sirtuin 1
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1
SUMO	Small ubiquitin-like modifier
TC-NER	Transcription-coupled nucleotide excision repair
TDG	Thymine-DNA glycosylase
TFIIH	Transcription factor IIH
TGD	Transglutaminase-like domain
USP7	Ubiquitin-specific processing protease 7
UV	Ultraviolet
VCP	Valosin-containing protein
XP	Xeroderma pigmentosum

Introduction

Living organisms are relentlessly challenged by exogenous and endogenous DNA-damaging agents that threaten genome integrity. Prominent types of DNA damage are "bulky" lesions consisting of base adducts or intrastrand crosslinks that destabilize complementary base pairing in the double helix. Such base pair-disrupting injuries arise from chemical carcinogens such as polycyclic aromatic hydrocarbons forming covalent base adducts [1], reactive drugs like cisplatin generating crosslinks between adjacent bases [2] or by-products of cellular metabolism including oxygen radicals yielding cyclodeoxynucleosides [3, 4]. The most commonplace lesions derive from exposure to the ultraviolet (UV) range of natural sunlight or artificial radiation sources, which induce crosslinks between neighboring pyrimidines, i.e., cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) [5]. If not promptly removed by DNA repair, these UV crosslinks like other bulky lesions interfere with transcription [6], DNA replication or cell cycle [7], and cause mutations and chromosomal aberrations that culminate in cancer as well as accelerated aging (reviewed by [8]). In particular, the incidence of skin cancer continues to increase, and thus remains a public health concern, despite widespread awareness that sunlight is the major risk factor for cutaneous malignancies [9, 10]. This review is focused on recent advances in our knowledge of how XPC protein carries out its DNA quality surveillance preventing sunlight-induced skin cancer. Since the discovery that DNA repair of UV damage critically depends on post-translational protein modifications [11, 12], it has become increasingly clear that multiple polypeptide modifiers control the pleiotropic activity of this versatile sensor of DNA integrity.

Excision repair of bulky DNA lesions

Nucleotide excision repair (NER) is the DNA repair system that removes bulky base lesions induced by chemical carcinogens, DNA-reactive drugs, by-products of aerobic metabolism or UV light. Being caused by various DNA-damaging agents, these NER substrates are structurally diverse, but always limited to one DNA strand. The cut-and-patch NER machinery operates by cleaving this damaged strand on either side of the injury, thereby excising the lesion as part of 24–32-nucleotide-long single-stranded segments [13, 14].

Depending on the context of its occurrence, DNA damage is detected by two alternative routes. In the transcription-coupled sub-pathway (TC-NER), damage is first sensed when the RNA polymerase II complex encounters obstructing base lesions during transcription [15]. This molecular collision with roadblocks triggers a reaction that is not yet fully understood, but eventually promotes the accelerated removal of base lesions from the transcribed strand of active genes (reviewed by [8, 16, 17]). On the other hand, bulky DNA lesions anywhere in the genome are detected, independently of RNA polymerase II, by a more general sub-pathway known as global-genome NER (GG-NER; reviewed by [18]). Defects in GG-NER result in the xeroderma pigmentosum (XP) syndrome, a devastating cancer-prone condition characterized by photosensitivity, severe sunburns and freckling, solar keratosis and an over 1,000-fold increased risk of sunlight-induced skin cancer [19]. XP patients also have a higher propensity of developing internal tumors attributable to chemical carcinogens or reactive oxygen species [20]. These patients are classified into different genetic complementation groups (from XPA to XPG) caused by mutations in the respective seven NER genes [21]. An eighth complementation group (XP-V) presents a variant form resulting from mutations in the gene coding for DNA polymerase η, which is responsible for the error-free bypass of UV lesions during DNA replication [22].

Core GG-NER machinery

A key feature of the GG-NER pathway is that it takes only a limited set of proteins to recognize and repair an extraordinarily wide spectrum of bulky lesions. Inducing tiny spots of UV damage in cell nuclei by irradiation through micropore filters is a frequently adopted strategy to study the intracellular trafficking of these GG-NER factors. In combination with biochemical reconstitution assays, this method demonstrated that locating bulky lesions depends on a heterotrimeric factor composed of xeroderma

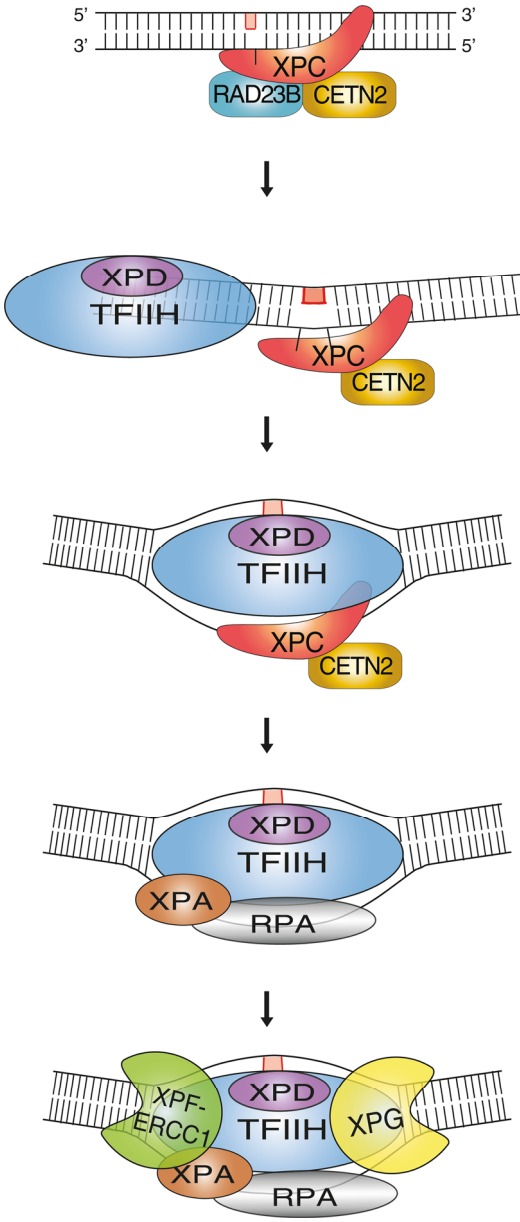

Fig. 1 Initiation of GG-NER activity by the heterotrimeric XPC complex. The XPC subunit is a thermodynamic sensor that recognizes base pair destabilizations of the DNA double helix caused by bulky lesions such as UV light-induced 6-4PPs or carcinogen-DNA adducts (symbolized by the *red rectangle* in the upper damaged strand). Briefly, the GG-NER reaction proceeds by a stepwise mechanism initiated by the trimeric XPC-RAD23B-CETN2 complex, which binds to ruptured base pairs and extends the local melting of DNA by flipping-out two nucleotides of the undamaged strand opposite to bulky lesions. After this initial sensing of damaged sites, the XPC subunit mediates the recruitment of XPD as part of the multimeric TFIIH complex. The DNA helicase activity of XPD is exploited to scan the damaged strand and, after reaching the injured base, this tracking enzyme forms a long-lived demarcation complex with the DNA duplex being unwound around the lesion. The single-stranded configuration of DNA in this intermediate is stabilized by RPA, which together with XPA positions the two structure-specific endonuclease "scissors" (XPF-ERCC1 and XPG) in a way that they cut the damaged strand at each Y-shaped double-stranded to single-stranded DNA junction. This dual cleavage results in the removal of bulky lesions in the form of oligonucleotide segments with a length of 24–32 residues. For the special case of CPDs, this GG-NER system needs the assistance of the DDB2 damage detector for substrate recognition

pigmentosum group C (XPC; [23, 24]) one of two human RAD23 homologs (predominantly RAD23B; [25]) and centrin 2 (CETN2; [26–28]). The DNA-binding and lesion recognition activity of this heterotrimeric complex resides entirely within the XPC subunit. The contribution of RAD23B (a 26S proteasome-interacting factor that escapes proteolytic degradation) and CETN2 (a calcium-binding protein also found in centrosomes) is to protect XPC from degradation and support its proper folding necessary to achieve optimal DNA-binding affinity [25, 29, 30]. The RAD23B partner supports the recognition of damaged DNA by XPC protein [31, 32] but is readily released once XPC associates with DNA lesions [32, 33]. On the other hand, CETN2 may remain associated with target sites, while still in complex with XPC, and facilitate downstream recognition steps [34].

The XPC-CETN2 heterodimer bound to DNA substrates forms a recruiting platform for transcription factor IIH (TFIIH; Fig. 1). This 10-subunit complex comprises the XPD helicase, which separates complementary strands in duplex DNA to generate an unwound configuration of about 25 nucleotides around the lesion [35, 36]. The resulting unwound intermediate is stabilized by XPA in conjunction with replication protein A (RPA), and the damaged strand is cut by structure-specific endonucleases at the double-stranded to single-stranded DNA junctions on each side of the lesion [35, 37]. Incision on the 5′ side is carried out by a heterodimer consisting of XPF and excision repair cross-complementing 1 (ERCC1), followed by the incision on the 3′ side through XPG [38]. Once the excised segment harboring the lesion is released, the resulting single-stranded gap is filled by DNA repair synthesis through the action of DNA polymerases δ, ε or κ [39]. Finally, full helix integrity is restored by DNA ligase I and DNA ligase IIIα that seal the nicks [40, 41].

Molecular structure of the multi-domain XPC sensor

The human *XPC* gene is located on chromosome 3, consists of 16 exons and codes for a protein of 940 amino acids [42]. The protein contains domains for binding to both DNA [43–45] and many protein partners (Fig. 2a). To serve as a common initiator of GG-NER activity, XPC

protein must be able to sense a wide variety of chemically unrelated DNA lesions. As these different substrates share no chemical motif that would support a canonical "lock-and-key" recognition mechanism, it was a major challenge to understand how this promiscuous sensor inspects the DNA double helix for a broad lesion spectrum.

A first insight towards solving this substrate versatility enigma came from a comparison of amino acid sequences indicating that human XPC displays short regions of homology with single-stranded DNA-binding domains of RPA and breast cancer 2 (BRCA2). This homology suggested that XPC protein is able to detect the local single-stranded character of DNA containing base pair-disrupting lesions [46]. Biochemical experiments demonstrated that XPC indeed exhibits a binding preference for single-stranded oligonucleotides, or double-stranded DNA with single-stranded overhangs, over duplex counterparts [45, 47, 48]. It was also observed that DNA lesions induced by UV or cisplatin within single-stranded DNA reduce XPC binding, indicating that XPC protein may avoid contacting damaged nucleotides. This aversion for damaged nucleotides, together with the preference for single-stranded DNA elements, suggested an indirect sensing mechanism by which XPC protein recognizes unpaired nucleotides in the undamaged strand, thus exploiting a generic attribute of damaged DNA featuring compromised base pairing [46, 49].

This unique mode of action was confirmed when a high-resolution structure of the evolutionarily conserved homolog from *Saccharomyces cerevisiae* (Rad4 protein) came to light [50]. The co-crystal structure with duplex DNA containing a model lesion shows that Rad4 deploys four core domains that bind to damaged DNA in two parts (Fig. 2b). One part is made up of a transglutaminase-like domain (TGD) and a β-hairpin domain (BHD1), which together associate with an 11-base pair duplex segment flanking the lesion. A second part entails two other β-hairpin domains (BHD2 and BHD3) that bind to a 4-nucleotide segment containing the lesion. In this interaction, the damage-containing base pairs are dislocated from the duplex inducing a flipped-out configuration, which we refer to as the "open" conformation. The BHD2–BHD3 domains embrace the nucleotides on the undamaged strand but not the damaged ones. Furthermore, a long β-hairpin finger protruding from BHD3 is inserted into the DNA, stabilizing the gap created from the flipped-out nucleotides.

In summary, biochemical and structural analyses revealed that the exquisite substrate versatility of yeast Rad4 and its human homolog XPC is achieved by an entirely indirect readout strategy that senses mis- or unpaired bases opposite to a bulky lesion in DNA duplexes. This unprecedented mechanism defies the traditional "fitting glove" [51] or "fitting shoe" strategy [52] whereby lesion recognition takes place through close interactions between a dedicated protein pocket and damaged nucleotide moieties, as shown for many DNA glycosylases participating in base-excision repair (BER). In GG-NER, DNA lesions are instead located by first detecting thermodynamic destabilizations inducing a local single-stranded character. The advantage of this indirect strategy by XPC/Rad4 is that the range of DNA damages sensed for further processing is greatly broadened.

Interactome of the XPC sensor

In addition to being involved in the association with DNA, the TGD domain is also required for the interaction between Rad4 and Rad23 [50], or between the respective human homologs XPC and RAD23B (see Fig. 2a). Part of the human TGD also interacts with XPA protein [53]. Another partner, known as DDB2 (for *D*amaged *D*NA-*B*inding 2; encoded by the *XPE* gene) appeared more recently in evolution and does not exist in lower eukaryotes like yeast. However, a transient interaction between DDB2 and XPC is pivotal for the processing of CPDs in mammals and the corresponding interaction domain has been mapped to the TGD and BHD1 domains [33]. Besides these central DNA-, RAD23B-, XPA- and DDB2-binding regions, residues 847–863 in the carboxy terminus of XPC form an α-helix that binds tightly to CETN2 [27, 54]. Residues 816-940 in this carboxy-terminal region as well as a portion of the amino-terminal region around residue 334 associate with two distinct subunits (p62 and XPB) of the TFIIH complex [55–57]. In addition, XPC protein interacts with different DNA glycosylases and the Oct4-Sox2 activator of pluripotency (see below). Recently, a high-throughput two-hybrid screen revealed 49 additional potential interactors with roles in DNA synthesis, proteolysis, post-translational modification including the OTU deubiquitinase 4 (OTUD4), transcription, signal transduction and metabolism. However, so far only the association with OTUD4 has been validated by immunoprecipitation [58]. There is also a biochemically proven interaction between human XPC and the USP7 deubiquitinase (for Ubiquitin-Specific-processing Protease 7) [59].

Search for bulky DNA lesions in the genome

The next challenging question is how the XPC complex scans the genome and succeeds in finding rare lesions with disrupted base pairs within 6.4 billion base pairs of native DNA. Fluorescence-based imaging methods provided a real-time strategy to track the mobility of repair proteins at work in their physiologic milieu in living cells. One main

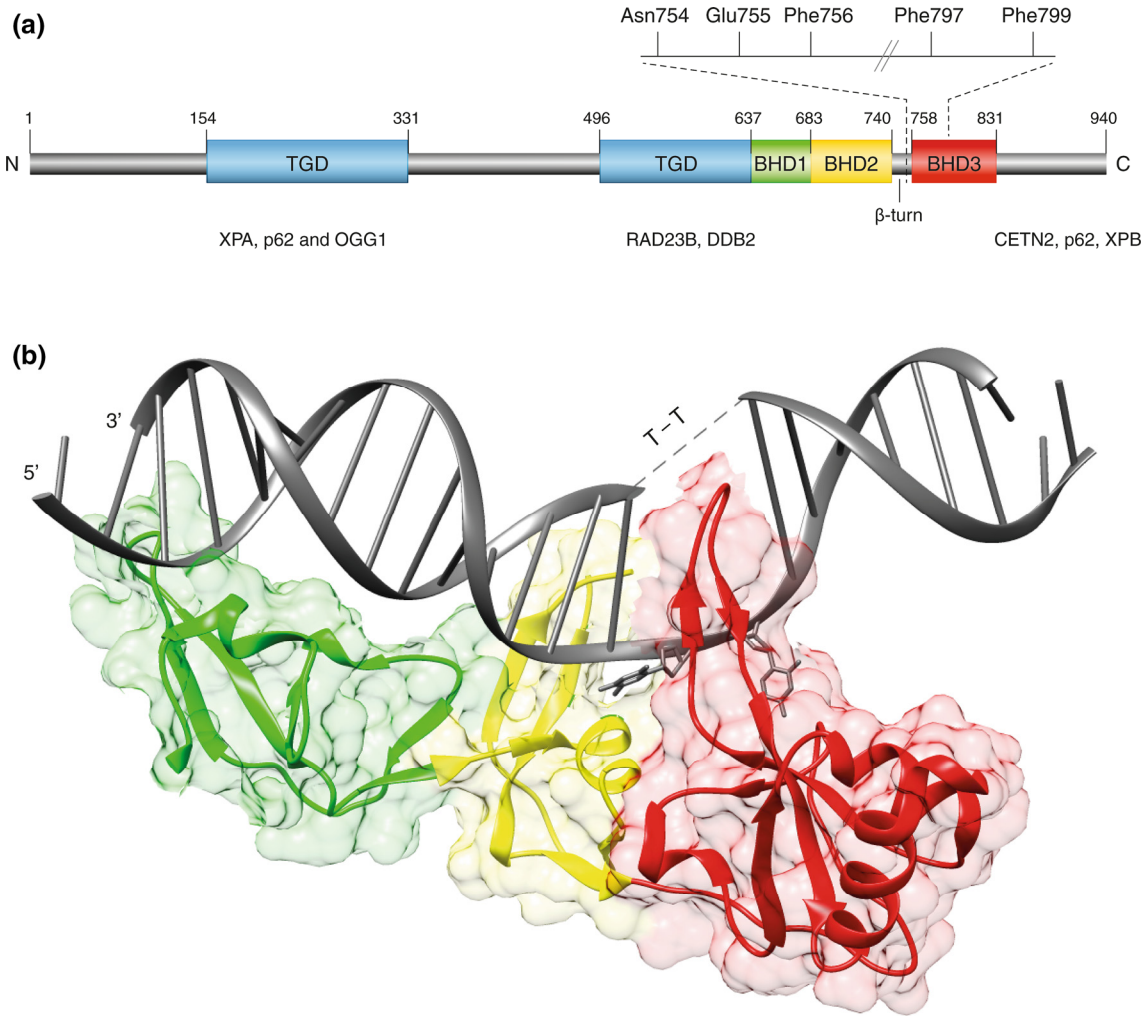

Fig. 2 Modular structure of the XPC protein. **a** Domain map of human XPC protein highlighting the transglutaminase-like domain (TGD) and the three β-hairpin domains (BHD1-3) interacting with DNA. TGD and BHD1: in the crystal structure of the RAD4 homolog from *S. cerevisiae* [50], TGD region, in conjunction with BHD1, binds to 11 base pairs of double-stranded DNA flanking the lesion. BHD2 and β-turn subdomain: protein dynamics studies in human cells [62] indicate that BHD2 together with the β-turn detect unpaired bases in the damaged double helix. BHD3: XPC protein becomes anchored onto lesions sites by the intra-helical insertion of a long β-hairpin "finger" protruding from BHD3. DNA-attractive amino acids (Asn754, Phe756, Phe797, Phe799) and a DNA-repulsive residue (Glu755) are responsible for the sensing and flipping-out of unpaired bases in the undamaged strand opposite to bulky lesions. The aforementioned domains are also involved in interactions with various protein partners (XPA, p62, OGG1, RAD23B, DDB2, CETN2, and XPB). **b** Ribbon diagram of the BHD1-BHD3 region of RAD4 in complex with DNA containing a CPD (indicated by T–T) embedded in three consecutive base mismatches [50]. TGD region is not shown. *Green* BHD1; *yellow* BHD2; *red* BHD3; *gray* DNA. This structure reflects the stably damage-anchored protein. While the three mismatches were necessary to allow for the binding of RAD4 protein to DNA, there are no contacts between RAD4 and the two pyrimidines of the CPD lesion, which are disordered and concealed by the solvent. The figure was prepared with the Chimera extensible molecular modeling system, using the structure PDB 2QSG

application, fluorescence recovery after photobleaching (FRAP), showed that the movement of the XPC complex is slower than expected from its predicted diffusion rate. This low mobility indicated that the initial complex, unlike the downstream GG-NER factors, does not freely diffuse across the nucleoplasm but associates with native DNA in chromatin while searching for sites of base pair destabilization [60, 61].

The mechanism of DNA damage search was investigated by site-directed mutagenesis of a short β-turn subdomain situated at the transition between the BHD2 and BHD3 domains of human XPC (see Fig. 2a). This particular study focused on a DNA-repulsive glutamic acid residue at codon 755 that is evolutionary conserved and located between two amino acids that make contacts with DNA in the co-crystal of the Rad4 homolog. Conversion of

this negatively charged glutamic acid, which clashes with the negatively charged deoxyribose-phosphate backbone of DNA, to the positively charged lysine increased the binding of XPC protein to the double helix. It was noted by FRAP that this charge inversion is sufficient to increase the residence time of XPC on native DNA and, accordingly, decrease its ability to freely move across chromatin. This charge inversion also reduces GG-NER efficiency. The DNA-repulsive residue in this β-turn motif is, therefore, key to efficient genome surveillance, as it prevents XPC from residing too long at any given native DNA site [62].

The importance of preventing a prolonged residence of XPC during its damage search process became evident from recent structural studies of Rad4 bound to native DNA. A co-crystal of Rad4 with undamaged DNA was captured by covalently tethering a TGD residue to duplex DNA [63]. The resulting structure showed that immobilized Rad4 is able to flip-out undamaged nucleotides exactly as observed before on damaged DNA without tethering [50]. This finding demonstrates that, by allowing a prolonged residence on DNA, Rad4 would flip-out even thermodynamically stable nucleotide pairs. This conclusion in turn implies that the binding of XPC to lesions is accomplished not by differences in the most stable, DNA-bound structures between damaged and undamaged DNA (since there is no difference), but by the kinetic probability difference in flipping-out nucleotides before the protein diffuses away. In temperature-jump perturbation spectroscopy experiments, the Rad4-induced DNA opening took ~7 ms at base pair-destabilized target sites, but the same process is orders of magnitude slower on native base pairs [63]. Compared with the sub-millisecond residence time of Rad4 on undamaged DNA, this opening time is too long to result in proficient interactions. Such a kinetic gating mechanism excludes the opening of native DNA while selectively opening damaged sites exhibiting ruptured base pairs.

That the just described "interrogate-and-open" process also takes place in the genome of human cells had been tested using fluorescently labeled XPC truncates. The differential redistribution of truncation products to UV lesion spots revealed that BHD1 and BHD2, together with the β-turn subdomain, are sufficient to interrogate the DNA double helix for the presence of non-hydrogen-bonded bases. To further mature into an open and stable recruitment platform, XPC protein additionally needs the BHD3 domain, which promotes insertion into the double helix of its β-hairpin finger [62]. With this hairpin insertion, the sensor is stably anchored onto the opened DNA duplex displaying two fully flipped-out nucleotides, which allow for the recruitment of the TFIIH complex [64, 65]. To summarize, XPC quickly scans the double helix for base pair integrity before undergoing extensive interactions at destabilized targets to ultimately form the open conformation. This interrogate-and-open process enhances the efficiency of lesion recognition by obviating the futile flipping-out of undamaged base pairs present in large excess.

Demarcation of bulky DNA lesions for NER activity

It is now clear that XPC does not act as a canonical DNA damage reader but rather as a thermodynamic sensor of ruptured base pairs without making contacts with chemically modified residues. The true role of XPC is to start DNA damage recognition by recruiting the XPD helicase as part of the TFIIH complex [66, 67]. In detail, site-directed mutagenesis of the β-turn and BHD3 regions showed that XPC protein projects the conserved residues Asn754, Phe756 and Phe797 to encircle one non-hydrogen-bonded nucleotide in the undamaged strand opposite to bulky lesions. Extrusion of the adjacent undamaged nucleotide is induced by interactions with Phe797 and Phe799 upon the β-hairpin insertion. The significance of this double flipping-out was tested by complementing XP-C fibroblasts with expression constructs coding for wild-type or mutated XPC protein. Immunochemical analyses showed that a substitution of Phe799 to alanine was sufficient to inhibit recruitment of the TFIIH complex to spots of UV lesions, thus demonstrating that the flipping-out of two nucleotides from the undamaged strand is a crucial prerequisite for TFIIH loading [68].

The XPD subunit of TFIIH displays a 5′-3′ helicase activity that provides a directional tracking engine for the scanning of individual DNA strands. Major structural and functional features of this helicase were deduced from the analysis of homologous proteins in archaeal organisms [69–71]. Their crystal structure revealed that XPD consists of two helicase motor domains (HD1 and HD2), an iron–sulfur cluster (4Fe–4S) and an auxiliary Arch domain (Fig. 3a). During enzyme translocation driven by ATP hydrolysis, the 4Fe–4S cluster and Arch domain are thought to cooperate in separating the complementary strands of duplex DNA, in a way that one strand enters a narrow hole of the enzyme and then moves along an internal channel, while the opposing strand is displaced to the backside of the protein (Fig. 3b).

Another archaeal homolog from *Ferroplasma acidarmanus* was used to analyze how XPD responds to a CPD lesion located either in the translocated strand entering the helicase hole or in the displaced backside strand. Biochemical assays showed that the helicase activity was

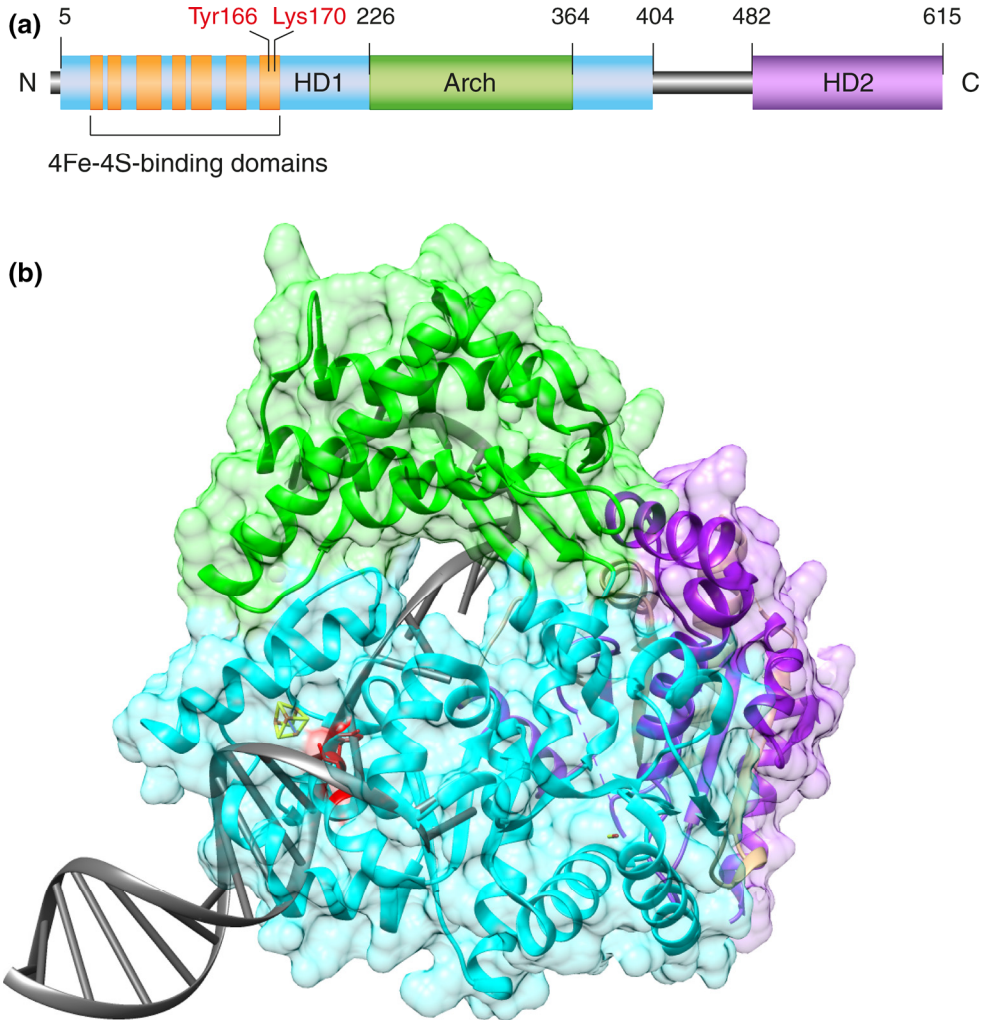

Fig. 3 Recognition of bulky DNA lesions by the XPD helicase. **a** Domain map of XPD protein from *Thermoplasma acidophilum* highlighting the helicase motor domains (HD1 and HD2), the Arch domain and the 4Fe–4S cluster. The evolutionary conserved amino acids Tyr166 and Lys170 (Tyr192 and Arg196 in the human homolog) are responsible for the recognition of damaged bases. **b** Ribbon diagram of the XPD helicase from *T. acidophilum* [152] modeled in complex with DNA to illustrate how one strand is thought to penetrate the central protein hole during the unwinding process. *Green* Arch domain; *light blue* HD1; *purple* HD2; *red* amino acids Tyr166 and Lys170; *gray* DNA. The residues Tyr166 and Lys170 (Tyr192 and Arg196 in the human homolog) are located in a strategic position near the central hole where they immobilize damaged bases just before they enter the protein tunnel [72]. The figure was prepared with the Chimera extensible molecular modeling system, using PDB accession codes 4A15 for the XPD [152] and 2P6R for the DNA [153]

blocked by a CPD and that this stalling of the helicase gives rise to a long-lived recognition complex that demarcates the lesion in the translocated but not in the displaced strand [67]. To understand this XPD-dependent scanning mechanism, amino acids in an evolutionary conserved nucleotide-binding surface near the entrance of the narrow hole were targeted by site-directed mutagenesis. Two of the resulting mutants retain ATPase and helicase activity, but in contrast to the wild-type control, are not arrested by a CPD while tracking along DNA. When the consequences of these mutations were tested in living cells, the two mutant XPD proteins failed to induce long-lived demarcation complexes at UV lesion spots and conferred defective GG-NER activity [72]. These reports prove that XPD is the *de facto* DNA damage recognition subunit in the NER pathway by trapping offending bases in a pocket of the enzyme surface just before they enter the narrow helicase hole (Fig. 3b).

Recruitment of further DNA repair pathways by the XPC sensor

In DNA-binding assays, the selectivity of the XPC sensor extends from bulky DNA lesions to certain smaller or "non-bulky" base modifications, including for example 8-oxo-guanine or methyl-formamidopyrimidine moieties, that are typical substrates of the BER pathway [73]. Accordingly, XPC protein is readily recruited to stripes of 8-oxo-guanines generated in the cell nuclei by low-energy irradiation with a 405-nm laser in the presence of a photosensitizing agent [74]. Other reports demonstrated that the XPC complex stimulates the activity of at least four distinct DNA glycosylases, which initiate BER reactions by cleaving *N*-glycosylic bonds from the deoxyribose-phosphate backbone, i.e., methylpurine-DNA glycosylase (MPG; [75]), thymine-DNA glycosylase (TDG; [76]), 8-oxo-guanine-DNA glycosylase 1 (OGG1; [77, 78]) and single-strand-selective monofunctional uracil-DNA

glycosylase 1 (SMUG1; [76]). Moreover, mouse and human cells lacking functional XPC are hypersensitive to the cytotoxic effects of oxidative agents and also display an increased sensitivity to etoposide, a topoisomerase II inhibitor that causes DNA breaks [79]. These different lines of evidence indicate that XPC might use its affinity for destabilized base pairs to serve not only as the initiator of GG-NER activity but also as a more general platform for the loading of multiple repair pathways, including BER and double-strand break repair systems, to damaged DNA carrying compound lesions.

XPC functions outside DNA repair

TFIIH was the first example of a functional link between the NER system and transcription. Indeed, the TFIIH complex was originally characterized as a basal transcription factor [80, 81] and the discovery that it is also an NER component came later when it was found that XPB and XPD, known to participate in DNA repair, represent ATPase and DNA helicase subunits of this multifunctional enzyme [82]. A second link between transcription and DNA repair was evidenced by the TC-NER sub-pathway, in which DNA damage encountered during transcription is removed through the action of many NER factors that are also involved in the GG-NER process. Although previously believed to act only as damage sensor in the GG-NER pathway, XPC was found to support transcription independently of its DNA repair function. Chromatin immunoprecipitation analyses indicated that XPC protein and, sequentially, downstream NER factors (XPA, RPA, XPG and XPF-ERCC1) home in on the promoter region of nuclear receptor-induced genes [83, 84]. Transcription inhibitors abolish this recruitment of GG-NER factors to active promoters but not to sites of DNA damage, demonstrating that their engagement with gene promoters is functionally distinct from the role in DNA repair. Cell lines with mutated XPC or XPA show reduced levels of mRNA expression from nuclear receptor-activated genes, implying that GG-NER factors optimize the efficiency of transcription. How exactly they co-activate the transcription machinery is not yet clear, but it has been observed that the presence of GG-NER factors in promoters is necessary to orchestrate a more permissive chromatin environment characterized by histone modification changes like H3K4 methylation, H3K9 de-methylation and H3K9 acetylation. One attractive hypothesis is, therefore, that XPC protein and accompanying NER factors exert a non-repair function by remodeling the epigenetic landscape to favor transcription [83]. Along this concept, it is tempting to propose that promoter occupancy by the GG-NER system may serve to install a more accessible chromatin environment regardless of whether the fate of the DNA substrate is to be repaired (in response to DNA damage) or to be transcribed (in response to promoter activation). That the GG-NER system may assemble in the absence of DNA lesions was confirmed by targeting XPC protein to undamaged genomic sites using a high-affinity lactose operator/repressor tethering system [85].

Yet another non-repair function of XPC emerged from the search for transcriptional co-factors that potentiate the Oct4- and Sox2-dependent expression of the *Nanog* pluripotency gene, which is needed for self-renewal of embryonic stem cells as well as for the reacquisition of stem cell-like properties. Using a defined in vitro transcription system, the XPC complex was identified as a co-activator of *Nanog* expression that interacts directly with the Oct4/Sox2 dimer [86]. This unexpected co-activator role was further tested by RNA interference of XPC, RAD23B and CETN2 expression in mouse embryonic stem cells. Down-regulation of the trimeric XPC complex triggered stem cell apoptosis, thus supporting the notion that this factor promotes pluripotency and self-renewal. Moreover, depletion of XPC or RAD23B compromised the induction of pluripotent stem cells from differentiated fibroblasts [86]. Notably, the XPC complex was still capable of co-activating *Nanog* transcription even if XPC contained a mutation (Trp690Ser) that abrogates binding to DNA or a truncation at position 813 that abrogates its interaction with TFIIH ([87]; see domain map in Fig. 2a). Another study even showed that the entire carboxy-terminal region of XPC is dispensable for the transcriptional activity of Oct4-Sox2. In this case, the *XPC* gene of mouse embryonic stem cells was down sized to the first 8 exons, which eliminates a large portion of the coding sequence, from residue 326 to the C terminus, but without compromising pluripotency [88]. However, the expression and stability of the expected amino-terminal XPC fragment of 325 amino acids had not been confirmed. Also, it is not yet possible to reconcile the finding that the XPC complex adopts a role during transcription, in both stem and somatic cells, with the fact that mice lacking the *XPC* gene show no overt developmental defects [89].

Control of *XPC* expression and cellular localization

In view of its diverse actions as a DNA quality sensor that interrogates the native double helix, permanently scrutinizing base pair integrity, associates with multiple DNA repair systems and also carries out non-repair functions in transcription, it is not at all surprising to note that the cellular level, localization and activity of XPC protein must be kept under tight control. Circumstantial evidence

suggests that XPC protein cannot exist in nuclei at high steady-state levels and, therefore, its expression and intracellular concentration must be tuned in accordance to the needs imposed by DNA damage or ongoing transcription. For example, even low expression of the yeast homolog Rad4 interferes with cell growth in *Escherichia coli* [90, 91] and, similarly, microinjection of complementary DNA coding for XPC and RAD23B proteins into human fibroblasts led to cytotoxicity [25]. Finally, it was shown that a faulty regulation of XPC homeostasis causes excessive chromosomal aberrations following UV exposure [92].

Under steady-state conditions in the absence of genotoxic stress, transcription of the *XPC* gene is down regulated by the E2F4-p130 repressor [93]. This transcriptional inhibition is relieved, on the one hand, by Sirtuin 1 (SIRT1), an NAD-dependent deacetylase that induces *XPC* expression by preventing nuclear localization of the E2F4-p130 repressor [94]. The transcriptional inhibition by E2F4-p130 is also relieved by the tumor suppressor ARF (for *A*lternative *R*eading *F*rame), which diminishes the binding of E2F4-p130 to regulatory sequences in the *XPC* gene promoter [95]. In response to UV light, ionizing radiation or alkylating agents, the *XPC* gene is induced by the tumor suppressor p53 [96, 97]. Using chromatin immunoprecipitation assays, a functional p53 binding sequence was identified within the *XPC* gene in an unusual location at the translational start site [98, 99]. Based on the finding that BRCA1 represents another positive transcriptional regulator of the human *XPC* gene, a sequential scenario has been proposed for an involvement of *XPC* in the progression of breast or ovarian cancer, where the loss of BRCA1 restricts the initiation of GG-NER activity by XPC protein and, therefore, causes an accumulation of DNA damage and mutations in the *p53* gene, which in turn leads to an even more pronounced GG-NER defect and genome instability [100].

The intracellular localization of XPC protein is influenced by a DNA damage-sensitive cytoplasmic–nuclear shuttling system. Under unchallenged conditions, XPC continuously shuttles between the cytoplasm and the nucleus driven by a balanced effect of nuclear localization and nuclear export signals in its amino acid sequence. Upon genotoxic stress for example by inflicting UV radiation, there is a shift in this cytoplasmic–nuclear balance towards higher XPC concentrations in the nucleus [25, 60]. The molecular mechanism underlying this nuclear retention in response to DNA damage is not yet understood, but polypeptide modifiers like ubiquitin or SUMO (for *S*mall *U*biquitin-like *MO*difier) have been implicated in the spatiotemporal regulation of XPC protein (see below). It is likely that increased nuclear XPC levels, achieved by enhanced expression as well as increased nuclear retention and reduced degradation, are necessary to optimize the detection of those lesions that are refractory to recognition or less accessible in densely condensed chromatin.

Support for the XPC sensor by the DDB2 detector

Exposure to UV radiation induces CPDs and 6-4PPs in a ratio of 3:1. These pyrimidine dimers differ in their biophysical properties and genomic distribution: CPDs cause relatively minor base pair destabilizations in duplex DNA compared to 6-4PPs [101–103]. Additionally, CPDs arise evenly across chromatin, whereas 6-4PPs are formed primarily in linker DNA rather than in nucleosome cores [104–106]. Because CPDs are removed at slower rates than 6-4PPs, they display a higher mutagenic potential and are responsible for most adverse effects of UV radiation ranging from sunburns to skin aging and cancer [107, 108].

Despite being the repair initiator for all bulky DNA lesions including CPDs, purified XPC protein does not bind CPDs with any measurable selectivity [43, 47, 109, 110]. This lack of specificity for CPDs is compensated by DDB2 (the factor mutated in XP-E patients; [111, 112]) whose transcription is also induced by the p53 and BRCA1 tumor suppressors, as seen for the *XPC* gene [113, 114]. Unlike XPC, which functions as a general sensor of helix disruptions independently of the nature of the offending lesion, DDB2 is specialized on the recognition of CPDs and 6-4PPs [115]. Crystal structures of DDB2 revealed a binding pocket, in the center of its β-propeller architecture, that is tailored towards high-affinity binding of CPDs and 6-4PPs while excluding larger base adducts (Fig. 4a; [116–118]).

The absence of a functional DDB2 protein in XP-E cells nearly abolishes the excision of CPDs although the repair of 6-4PPs is only slightly reduced [113, 119]. A widely accepted model is that DDB2 recognizes the CPDs and then delivers them to XPC for initiation of GG-NER activity [115, 120, 121]. However, the precise handover mechanism remained elusive for a long time because, in biochemical assays, purified DDB2 and XPC proteins compete directly for UV lesions and it was not possible to isolate stable intermediates where these two factors bind together to one damaged DNA site [122]. An explanation for this failure to isolate ternary DDB2-XPC-DNA intermediates came from the individual co-crystals. Each structure showed a DNA kink at the lesion site, but the kinks were in diametrically opposite directions when compared with each other. Moreover, both DDB2 and XPC insert a β-hairpin finger into the double helix, such that one would clash with the concomitant binding of the other [50, 116, 117].

The mechanism of substrate handover from DDB2 to XPC was eventually investigated using methods that detect short-lived interactions in the chromatin environment,

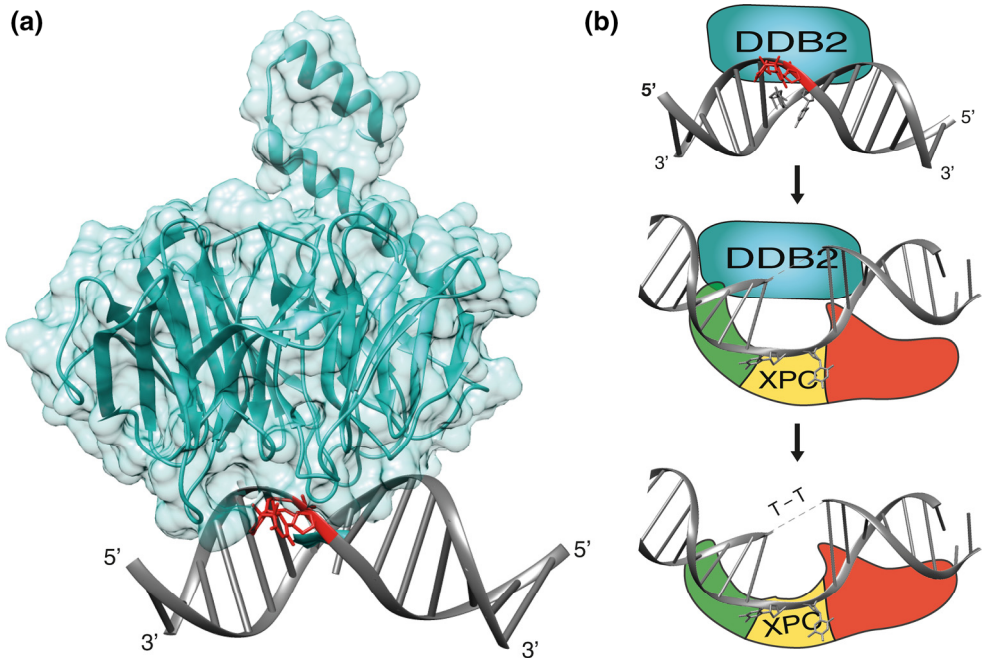

Fig. 4 Assistance by the DDB2 damage detector. **a** Ribbon representation of DDB2 from zebrafish in complex with double-stranded DNA containing a CPD lesion [117]. *Blue* DDB2: *gray* DNA; *red* CPD. The figure was prepared with the Chimera extensible molecular modeling system, using PDB accession code 4A09. **b** Recognition of a CPD by the DDB2 damage detector and handover of the lesion to the XPC partner. Binding of DDB2 to the UV lesion in DNA triggers an interaction with the BHD1 fold of XPC protein. This transient association of DDB2 with XPC at lesion sites facilitates insertion of the long β-hairpin "finger" of XPC into the DNA duplex, followed by the release of DDB2 [33]. A direct substrate handover from DDB2 to XPC is required for the excision of CPDs that, on their own, induce minimal base pair disruption and, hence, are not recognizable by XPC alone. *Light blue* DDB2; *Green*, *yellow* and *red* BHD1, BHD2 and BHD3 folds of XPC, respectively

including in situ domain mapping at spots of UV lesions and FRAP on local damage (FRAP-LD), combined with biochemical assays using isolated XPC domains [33]. These studies demonstrated that XPC lends two of its DNA-binding domains (TGD and BHD1) to interact transiently with DDB2 bound to a CPD or 6-4PP lesion. This short-lived intermediate at the site of damage facilitates the insertion of the β-hairpin of BHD3 into the DNA duplex, thereby pulling DDB2 away (Fig. 4b). It is important to point out that the β-hairpin insertion by XPC involves an energetic cost as it occurs by local breakage of stacking interactions and hydrogen bonds between the bases. Though 6-4PPs facilitate this insertion by lowering the melting temperature at the site of damage, XPC protein depends on DDB2 to interact productively with CPD sites, thus explaining the defect of XP-E cells in repairing CPD lesions.

Post-translational modification of XPC with polypeptide modifiers

In addition to serving as a direct UV lesion detector, the DDB2 protein exists in complex with the adaptor protein DDB1 that recruits the cullin 4A (CUL4A) scaffold and the RING finger protein ROC1 (for Regulator of Cullins 1), which together form the $CRL4^{DDB2}$ ubiquitin ligase. By mediating the covalent attachment of one or more 8-kDa ubiquitin moieties to target proteins [11], this cullin-type ligase provides an additional layer in the fine-tuning of GG-NER activity. Under unchallenged conditions, the $CRL4^{DDB2}$ ubiquitin ligase is maintained in an inactive state by a further association with the COP9 signalosome, a multi-subunit regulatory protease [117]. Upon detection of UV lesions by DDB2, the COP9 signalosome is released, allowing for the modification of CUL4A with the ubiquitin-like modifier NEDD8, which in turn activates the ubiquitin ligase to modify substrates located within approximately 100 Å, generating Lys48-linked ubiquitin chains [116]. The main ubiquitination targets include histones H2A, H3 and H4 as well as DDB2 itself and its interaction partner XPC [12, 123–126].

Upon UV exposure, the $CRL4^{DDB2}$-mediated ubiquitination of histones is thought to help opening chromatin and facilitate the access of repair systems to damaged DNA [125], but this hypothesis is challenged by the finding that CUL4A conditional-knockout mice show higher rather than reduced GG-NER activity [127]. It is, therefore, possible that the $CRL4^{DDB2}$ ligase may have a more

specific regulatory role with functional nuances depending on the organism (human or mouse), cell type or genetic background. There is, however, concordance on the finding that the auto-ubiquitination of DDB2 not only abrogates its DNA-binding ability but also triggers a rapid degradation by the 26S proteasome [12]. The same $CRL4^{DDB2}$ ligase ubiquitinates XPC but, in this case, XPC retains its DNA-binding activity and is partially protected from proteasomal destruction (see below). XPC protein is additionally modified with Lys63-linked ubiquitin chains by a separate ligase known as RNF111/Arkadia [128]. This further ubiquitination is contingent on a prior UV-dependent modification of XPC with SUMO [129]. Thus, GG-NER activity in response to UV damage is controlled by a variety of polypeptide modifiers, including SUMO, Lys48- and Lys63-linked ubiquitin chains, which decorate XPC protein at 15 or more distinct modification sites. Interestingly, down-regulation of $CRL4^{DDB2}$ or Arkadia have opposite effects by inhibiting or stimulating, respectively, the accumulation of XPC at UV damage spots, indicating that Lys48-linked ubiquitin chains (produced by $CRL4^{DDB2}$) and Lys63-linked counterparts (produced by Arkadia) exhibit diverging modulating roles [128, 130, 131].

Control of XPC dynamics in the chromatin of UV-irradiated cells

The packaging of genomic DNA is a compromise between two opposite needs: the DNA must be compacted to fit into the nucleus but still remain accessible to biological processes including DNA repair. To accomplish this dual requirement, DNA assembles with histones to generate a condensed array structure whose basic unit is the nucleosome (reviewed by [132, 133]). Each nucleosome repeat consists of a core particle, where 147 base pairs of the DNA duplex are wrapped around an octamer of core histones (two each of H2A, H2B, H3 and H4), separated by linker DNA of variable lengths. In higher eukaryotes, additional levels of packaging are achieved by interactions of histone H1 with linker DNA.

It is fundamentally important to view the regulatory role of polypeptide modifiers during repair within this native chromatin context. A conceptually new contribution to understanding the function of $CRL4^{DDB2}$-mediated ubiquitination came from the enzymatic dissection of chromatin by micrococcal nuclease (MNase). This enzyme digests DNA in the accessible linker more easily than that in nucleosome cores. Therefore, MNase treatments generate a soluble supernatant containing non-histone proteins that, before digestion, were associated with inter-nucleosomal linkers (amounting to ~35 % of total genomic DNA). Even at saturating enzyme levels, however, MNase digestions leave behind the vast majority of nucleosome core particles (amounting to ~60 % of total DNA) in the form of a densely packed and insoluble nucleoprotein fraction [134].

Two previous findings led us to predict that the $CRL4^{DDB2}$ activity in response to UV irradiation is not uniformly distributed across this nucleosome landscape consisting of core particles divided by linker segments. First, DNA-binding assays demonstrated that DDB2, the UV lesion-binding subunit of $CRL4^{DDB2}$, associates with a 15-fold higher affinity with 6-4PPs ($K_a = 1.5 \times 10^9\ M^{-1}$) compared to CPDs ($K_a = 1 \times 10^8\ M^{-1}$) [110, 135]. Second, 6-4PPs are formed mainly in linker DNA [104, 106]. For these reasons, it was not surprising to observe that, immediately upon UV irradiation, DDB2 associates predominantly with MNase-hypersensitive, highly accessible inter-nucleosomal sites [33]. On the other hand, it was generally thought that XPC on its own is unable to interact with DNA wrapped onto nucleosome cores [44] but, against this prevailing concept, the MNase probing revealed that XPC protein associates rather evenly with MNase-resistant, densely packed nucleosomes and MNase-sensitive inter-nucleosomal DNA. Upon UV irradiation, the binding of XPC to MNase-resistant core particles is further enhanced [33] and this finding is supported by cell imaging studies indicating that XPC is recruited to the condensed chromatin of interphase nuclei [136] and to condensed mitotic chromosomes [60].

In accordance with the preference of DDB2 for UV lesions located in inter-nucleosomal DNA, the entire $CRL4^{DDB2}$ ubiquitin ligase complex is recruited mainly to these accessible sites after UV irradiation. The consequence of this partitioning is that essentially only the fraction of XPC bound to inter-nucleosomal DNA is ubiquitinated whereas XPC bound to condensed core particles avoids ubiquitination [33]. The role of $CRL4^{DDB2}$ was then challenged using the following strategies: down-regulation of either DDB2 or CUL4A using RNA interference, depletion of nuclear ubiquitin using the proteasome inhibitor MG132 or blockage of ubiquitination using the small-molecule E1 inhibitor PYR-41 [137]. Alternatively, the ubiquitination of XPC was suppressed in mouse cells expressing a temperature-sensitive E1 mutant or an XPC-GFP fusion protein that is refractory to ubiquitination. In all these cases, the XPC molecules remained without ubiquitin modifications and were nearly completely relocated to the fraction of packed nucleosome core particles [33]. We, thus, concluded that the $CRL4^{DDB2}$-mediated ubiquitination of XPC serves to retain XPC at inter-nucleosomal sites, representing DNA repair hotspots for the efficient recruitment of downstream NER factors and fast UV lesion excision (Fig. 5). In the absence of

$CRL4^{DDB2}$ activity, more XPC binds to CPDs located within nucleosome core particles that represent a less permissive chromatin environment with poor recruitment of downstream GG-NER factors and slow excision of UV lesions. The default-mode association of XPC with core particles, counteracted by $CRL4^{DDB2}$-mediated ubiquitination, contradicts a long-held notion derived from in vitro reconstitution experiments that nucleosomes pose a barrier to recognition of UV lesions by XPC [44, 138]. In summary, these studies showed that the $CRL4^{DDB2}$-mediated ubiquitination serves to establish a distinctive spatiotemporal distribution of the XPC sensor thereby optimizing the recruitment of downstream NER factors in mammalian chromatin (Fig. 5).

Narrow time window of XPC regulation by $CRL4^{DDB2}$

The nearly instantaneous auto-ubiquitination of DDB2 by $CRL4^{DDB2}$, and ensuing proteolytic degradation of DDB2, translates to an automatic time machine that restricts the ubiquitin ligase activity and its regulatory influence on the XPC partner, to a window of only few hours after UV irradiation. Due to DDB2 breakdown, the degree of XPC ubiquitination diminishes progressively and, as a consequence, this repair initiator relocates from inter-nucleosomal DNA to not yet repaired UV lesions, mainly CPDs, in nucleosome core particles [33]. The time window of this $CRL4^{DDB2}$ action may be prolonged by simultaneous post-translational modifications with poly(ADP-ribose) (PAR) occurring within seconds after UV exposure. Down-regulation of the enzyme poly(ADP-ribose) polymerase 1 (PARP1), by treatment with a small-molecule inhibitor or by RNA interference, reduced the PAR accumulation at UV damage spots and inhibited the excision of CPDs [139, 140]. One report proposes a scenario where PARP1 modifies DDB2 and thereby competes with concurrent ubiquitination, which results in enhanced stability and chromatin retention of the DDB2 subunit [139]. In another study, opposite effects were observed because PARP1 inhibition prevented ubiquitination and removal of DDB2 from chromatin, thus indicating that PARP1 stimulates the DDB2 turnover [140]. Regardless of how exactly PARP1

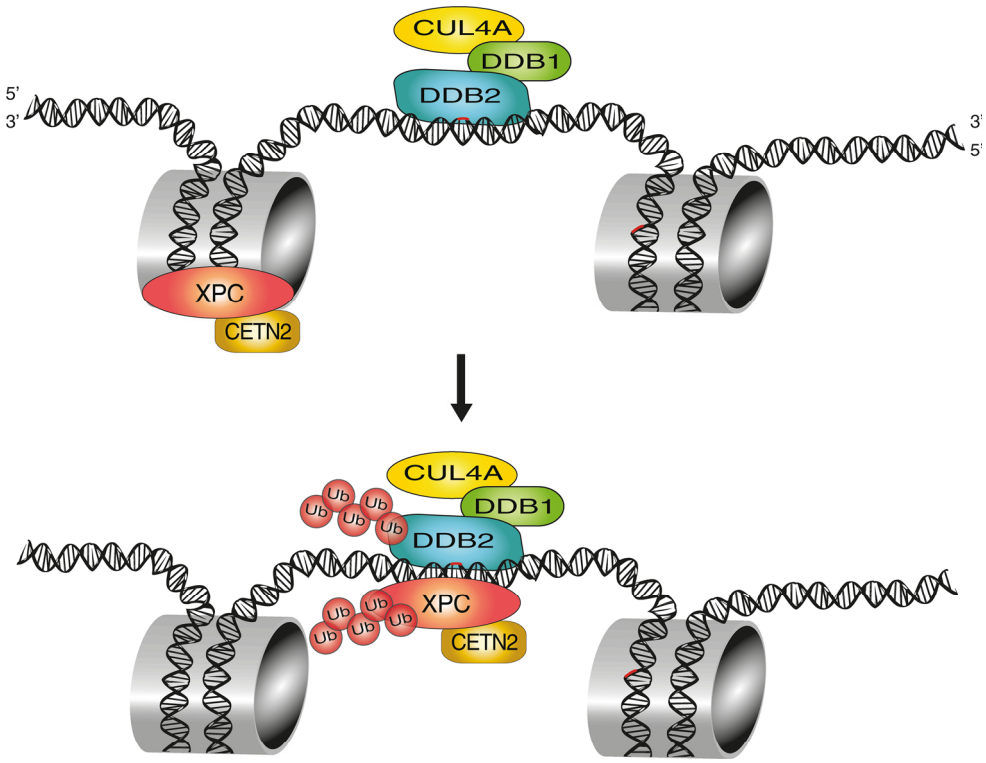

Fig. 5 Spatiotemporal control of XPC distribution in chromatin. The cullin-type $CRL4^{DDB2}$ ligase complex prioritizes the excision of UV lesions located in highly accessible chromatin sites. A preferential binding of the damage detector DDB2 to UV lesions in inter-nucleosomal DNA leads to the recruitment and ubiquitination of the XPC partner. This conjugation with polypeptide modifiers promotes the temporary retention of XPC at inter-nucleosomal sites, thus suppressing its constitutive association with nucleosome core particles. This transient ubiquitin code on XPC is necessary for the fast excision of UV lesions from inter-nucleosomal DNA. Thereafter, DDB2 is progressively degraded whereas XPC is de-ubiquitinated to allow for the recognition of UV lesions in nucleosome core particles [33]

impinges on the DDB2 half-life, the formation of PAR at sites of UV damage may generate a dynamic scaffold that promotes transient interactions of DDB2 with XPC and facilitates the recruitment of adjuvant factors that stimulate DNA repair like for example the ALC1 (for *A*mplified in *L*iver *C*ancer 1) chromatin remodeler [139] or histone-acetylating enzymes [141, 142].

Ubiquitin-dependent extraction of DDB2 and XPC from chromatin

Even though the DDB2 damage detector is needed for efficient excision of UV lesions, particularly CPDs, Lys48-linked ubiquitination triggers its degradation within few hours after exposure to UV light [123, 143]. It remained enigmatic why UV radiation induces the degradation of most DDB2 subunits well before excision of CPDs from the genome is completed. The actual scope of this apparently paradoxical breakdown of a DNA lesion detector remained unclear. There are also controversial findings as to whether XPC is partially degraded in response to UV damage [12, 25, 129].

Addressing these questions, it has been demonstrated that both DDB2 and XPC, once modified with Lys48 ubiquitin chains, become a substrate of the ubiquitin-selective p97 segregase, also known as valosin-containing protein (VCP) [92]. Individual p97 subunits assemble to form hexamers that convert ATP hydrolysis into mechanical force, which is used to extract ubiquitinated conjugates from cellular structures [144, 145]. The recognition of ubiquitinated DDB2 and XPC by p97 was demonstrated in situ on UV lesions spots in the nuclei of human cells, and confirmed biochemically by demonstrating that Lys48-ubiquitinated DDB2 and p97 reside in the same multiprotein complex. This recruitment of p97 to ubiquitinated DDB2 and XPC was shown to depend on various adapter proteins known to confer substrate specificity to the p97 segregase [146, 147].

The newly discovered involvement of p97 segregase activity in the GG-NER pathway provided an elegant strategy to test the consequences of an uncontrolled accumulation of DDB2 or XPC in chromatin. For that purpose, p97 activity was down regulated either by RNA interference or, alternatively, by mild overexpression of a dominant-negative mutant, which still binds ubiquitinated proteins but lacks segregase function and, consequently, remains trapped on ubiquitinated substrates [148]. With decreased p97 activity, there was excessive accumulation of both DDB2 and XPC in spots of UV lesions, indicative of an abnormal retention in UV-damaged chromatin, but without any increased recruitment of downstream NER factors like XPB (a component of the TFIIH complex) or ERCC1 (subunit of the ERCC1-XPF endonuclease complex). This down-regulation of p97 inhibited the UV-induced proteolytic clearance of DDB2 and also increased the level of ubiquitinated XPC. Unlike DDB2-ubiquitin conjugates, ubiquitinated XPC is processed in a p97-dependent manner by the USP7 deubiquitinase, thus restoring unmodified protein [59].

Despite their undisputed roles in the initiation of GG-NER activity, abnormally persisting DDB2 and XPC reduce the rate of UV lesion excision. This compromised DNA repair efficiency translates to hypersensitivity to UV radiation as well as enhanced chromosomal aberrations after UV exposure. Importantly, the genome instability observed in UV-irradiated cells after p97 depletion was reversed by concurrent down-regulation of DDB2 or XPC [92]. These findings suggested that the accumulation of either DDB2 or XPC is detrimental and that a tight control of their levels in chromatin is essential for genome stability. If this hypothesis were correct, then excessive expression of one of these factors would be sufficient to cause genome instability. In support of this intriguing concept, we observed that in a background of normal p97 activity, overexpression of wild-type DDB2 but not overexpression of a defective DDB2 mutant, inhibited the excision of UV lesions and enhanced the frequency of chromosomal aberrations after UV exposure. Double overexpression experiments involving both DDB2 and p97 demonstrated that increased levels of chromatin-bound DDB2 compromise genome stability only as long as they exceed the turnover capacity of the p97 segregase. Thus, a strict spatiotemporal control of the chromatin homeostasis of DDB2 and XPC by the p97 segregase is critical for efficient NER activity and a key function of the Lys48-linked ubiquitin modification of DDB2 and XPC is to prime these initial NER players for subsequent release from chromatin [92].

The paradigm of DDB2 homeostasis illustrates how both low and high levels of a DNA damage recognition factor impede repair and cause genome instability (Fig. 6). DDB2 stimulates the excision of UV lesions but, if bound to damaged chromatin in excess due to a failure in its extraction or degradation, this same sensor acquires genotoxic properties culminating in chromosomal aberrations. Evidently, DNA damage sensors such as DDB2 and XPC act as a double-edged sword as they trigger a beneficial defense but become unfavorable if allowed to accumulate in chromatin without control by the p97 segregase.

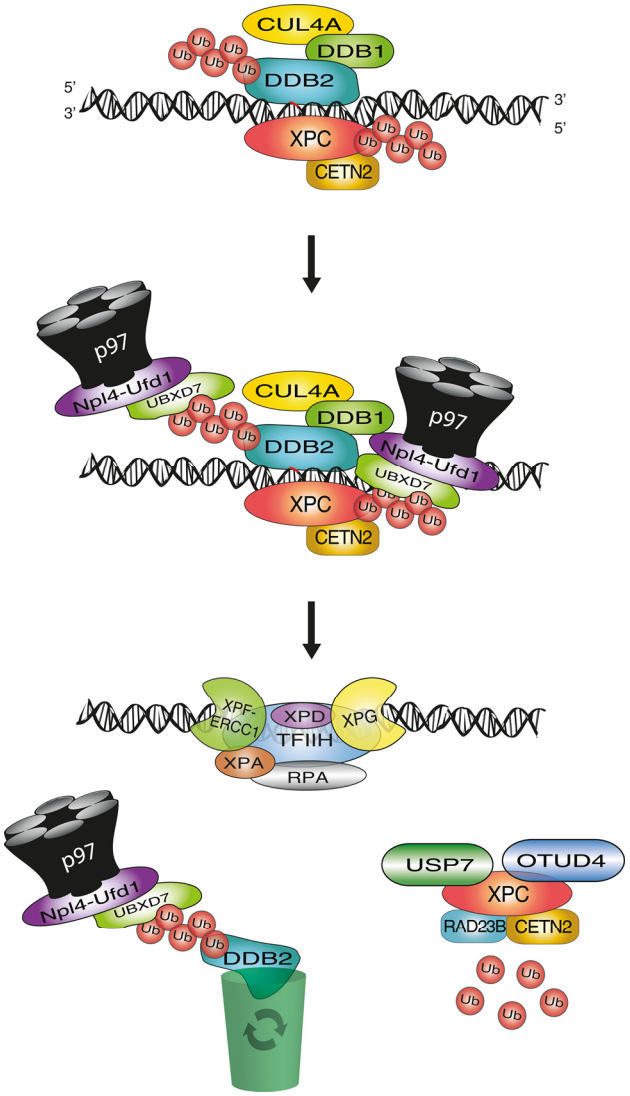

Fig. 6 Extraction of the UV detector DDB2 and damage sensor XPC from chromatin. The p97 segregase complex regulates GG-NER activity by removing ubiquitinated DDB2 and XPC from chromatin, thus favoring downstream recognition (by TFIIH) and excision steps (by XPF-ERCC1 and XPG). Ubiquitinated DDB2 is delivered to the proteasome system for degradation, whereas XPC is mostly recycled by de-ubiquitination through the action of USP7 or other de-ubiquitinating enzymes like OTUD4 [58, 59, 92]

Conclusion

The XPC complex functions as the general initiator of GG-NER activity by virtue of its ability to sense the presence of unpaired bases in double-stranded DNA and recruit the XPD verifier for subsequent bulky lesion confirmation. The clinical feature of a mutated *XPC* gene in xeroderma pigmentosum (hypersensitivity to UV radiation and skin cancer) highlights the extraordinary importance of this repair-initiating function for the excision of photodimers (CPDs and 6-4PPs) induced by sunlight exposure.

In a wider perspective, life on the planet Earth would not have been possible without the emergence of effective DNA repair mechanisms for the removal of UV photolesions. Indeed, most living organisms exposed at least transiently to sunlight possess a very rapid, highly efficient and safe enzymatic tool for the repair of photolesions in the form of DNA photolyases that, by light-driven catalysis, revert CPDs or 6-4PPs to pyrimidine monomers without excision of bases or any deoxyribose-phosphate residues [149, 150]. Unlike other animals, however, placental mammals are devoid of this simple light-driven DNA repair activity, presumably because they evolved from strictly nocturnal species originating from the Cretaceous era [151]. To finally return to a diurnal life under sunlight, placental mammals needed to "upgrade" their GG-NER pathway that constituted a hazardous backup system for the excision of base lesions refractory to photolyases or similarly innocuous reversal processes. In principle, many potential disadvantages are associated with implementation of the GG-NER system as the sole DNA repair defense against bulky UV lesions in placental mammals. First, CPDs would escape repair because the generic XPC sensor initiating GG-NER activity is not able to detect this most prevalent type of UV lesion. Second, sunlight-exposed skin cells would be faced with the uncoordinated cleavage of their DNA at thousands or more chromosomal sites nearly simultaneously, which would unavoidably threaten genome stability. Third, CPDs arise with a uniform pattern throughout the genome, including highly condensed sites that are poorly accessible and refractory to the assembly of GG-NER complexes.

Fascinating advances of the last decade in the field of GG-NER indicate that these aforementioned problems are countered inter alia by the following strategies. First, the accessory UV damage detector DDB2 attracts the XPC complex to CPDs that would otherwise remain unrecognized. Second, the repair-initiating activity of XPC is spatially regulated. By recruitment of the $CRL4^{DDB2}$ ligase mediating XPC ubiquitination, activity of the GG-NER pathway is in the beginning limited to highly accessible nucleosome-free sites that are easily amenable to the entire set of downstream excision factors, thus protecting more compacted chromatin localizations from accidental incisions that could trigger chromosome fragmentation. Third, the repair-initiating activity of XPC is temporally regulated. Through degradation induced by the $CRL4^{DDB2}$ ubiquitin ligase, the repair-stimulating activity of DDB2 is self-limiting and lasts only few hours after an acute dose of UV damage. Fourth, XPC is able to sense UV lesions within tightly packed nucleosomes and, by a not yet understood epigenetic mechanism affecting the local histone code, generates a more DNA repair-permissive chromatin landscape. This latter mechanism is also

employed for chromatin rearrangements occurring during transcriptional reprogramming of cells independently of DNA damage. Finally, rapid extraction of a surplus of ubiquitinated DDB2 and XPC from chromatin ensures optimal GG-NER activity and avoids molecular collisions with other ongoing processes like transcription or DNA replication. Only by adoption of these regulatory circuits during mammalian evolution, it has become possible to deploy the GG-NER pathway as the sole DNA repair system protecting from the mutagenic and carcinogenic effects of UV light.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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2 Aim of the Thesis

Nucleotide excision repair (NER) is a versatile DNA repair pathway that is able to remove DNA adducts as well as photoproducts induced by UV light and, as a consequence, of particular importance to avoid mutagenesis. Although the NER pathway has been extensively studied in mammals, yeast and bacteria, most of our knowledge on this process derives from work carried out in reconstituted *in vitro* systems. Many of these previous *in vitro* studies neglect or simplify the fact that DNA repair processes in eukaryotes do not take place on naked DNA but in the complex chromatin environment. Current models, known as unfold-repair-restore or also access-repair-restore mechanisms, address this problem but are largely unproven. These models predict that initial DNA repair steps include reactions that permit access of the repair machinery to DNA damage and that later steps restore the nucleosomal organization of the repaired DNA. Mechanistic details have been reported only for the late chromatin re-assembly steps.

The specific hypothesis behind the proposed project is that chromatin rearrangements takes place during the repair process. We postulated that *Chromatin Remodelling Complexes* (CRCs) and histone-modifying enzymes work in parallel to allow for NER activity in the carcinogen-damaged chromatin context.

The overall aim of my project was to understand how chromatin remodelling affects and modulate nucleotide excision repair activity. Specific aims of the project were (1) how is chromatin remodelling coordinated with the NER process? (2) at what particular step does chromatin remodelling influence the NER pathway, (3) What are the effects of impaired chromatin remodelling during the repair process.

Overall, this project was expected to shed light into the co-operation of chromatin remodelling and nucleotide excision repair.

3 Results

3.1 UV Lesion Verification by XPD in the Chromatin

Section 3.1 constitute an article (Mathieu *et al*, 2013) published 2013 in the journal *Current Biology* and is entitled:

DNA Quality Control by a Lesion Sensor Pocket of the Xeroderma Pigmentosum Group D Helicase Subunit of TFIIH

This paper describes the DNA lesions verification system in nucleotide excision repair by xeroderma pigmentosum group D. *N. Mathieu, N. Kaczmarek, P. Rüthemann, A. Luch and H. Nägeli* showed that XPD is able to scan and verify DNA modifications on specific strands during the unwinding process.

I designed, performed and analysed the following experiments in this study:

(1) Resolving the chromatin binding affinity of mutant XPD variants compared to the wildtype counterpart (**Fig. 3D**).

(2) Bioinformatical sequence alignment of XPD sequences of various species and annotations of protein domains (**Fig. Suppl. 1A**).

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Article

DNA Quality Control by a Lesion Sensor Pocket of the Xeroderma Pigmentosum Group D Helicase Subunit of TFIIH

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Summary

Background: Nucleotide excision repair is a versatile DNA repair reaction that removes bulky adducts generated by environmental mutagens such as the UV spectrum of sunlight or chemical carcinogens. Current multistep models of this excision repair pathway accommodate its broad substrate repertoire but fail to explain the stringent selectivity toward damaged nucleotides among excess native DNA. To understand the mechanism of bulky lesion recognition, we postulated that it is necessary to analyze the function of xeroderma pigmentosum group D (XPD) protein beyond its well-known role in the unwinding of double-stranded DNA.
Results: We engineered two new XPD mutants (Y192A and R196E), involving amino acid substitutions near its central protein pore, that confer defective DNA repair despite normal transcription. In situ fluorescence-based protein dynamics studies in living cells demonstrated that both new mutants were unable to recognize DNA damage and failed to form stable associations with lesion sites. However, when their biochemical properties were tested in the framework of an archaeal protein homolog, they both retained ATPase and DNA-unwinding activity. The outstanding difference versus the wild-type control was that their directional 5′–3′ translocation along DNA was not stopped by a bulky lesion, and moreover, they were unable to build long-lived demarcation complexes at damaged sites.
Conclusions: By uncoupling for the first time the unwinding and damage sensor activities of XPD, we describe an unprecedented genome quality control process whereby a recognition pocket near the central DNA helicase pore scans individual substrate strands to capture base adducts.

Introduction

A versatile DNA repair machinery known as nucleotide excision repair (NER) promotes genome stability by removing bulky base lesions induced by a diversity of genotoxic insults including the UV radiation of sunlight, chemical carcinogens, metabolic byproducts, and oxygen radicals [1–4]. This defense reaction consists of two subpathways. Global genome repair, which takes place across the entire genome, is initiated by the xeroderma pigmentosum group C (XPC) complex [5–8]. The second subpathway, transcription-coupled repair, is triggered by the stalling of RNA polymerase II and hence targets the transcribed strand of active genes [9, 10]. These two reaction branches converge into a common repair pathway with the recruitment of the multifunctional transcription factor IIH (TFIIH), whose two subunits with unwinding activity (xeroderma pigmentosum group B and D; XPB and XPD) are absolutely necessary to generate a melted DNA intermediate amenable to DNA damage excision [11–13]. At this stage of NER activity, XPB functions as an ATPase, whereas XPD also displays a processive 5′–3′ helicase action and hence provides a directional tracking engine [14, 15]. Mutations in the *XPD* gene give rise to hereditary disorders including xeroderma pigmentosum (XP), which predisposes to cancer, as well as Cockayne syndrome (CS) and trichothiodystrophy (TTD), characterized by developmental and neurologic deficits as well as traits of premature aging [16–18].

Although XPC protein, the initiator of global genome repair, is thought to detect distortions of the DNA double helix, it displays only a limited selectivity for many common NER substrates, including for example cyclobutane pyrimidine dimers (CPDs) induced by UV light [7, 19–21]. Particularly in the condensed chromatin context of living cells, XPC also associates extensively with the native double helix [22–24], thus highlighting its inability to clearly distinguish between damaged and undamaged DNA. Such findings raise the question of whether a downstream NER factor like TFIIH may be required to actually recognize or verify the presence of bulky DNA lesions before triggering the NER reaction [25–27]. Previous biochemical assays indeed suggest that the TFIIH complex may contribute to the search for bulky DNA damage [28]. However, it has never been shown conclusively whether damage recognition or verification by TFIIH, besides its unwinding function, is a true prerequisite for NER activity. Also, the mechanism of this proposed damage verification process has remained elusive.

Here, we tested the hypothesis that the directional tracking activity of the XPD subunit of TFIIH, besides its role in duplex unwinding, may serve as a molecular sensor to "read" the chemical integrity of DNA. This hypothesis was prompted by crystal structure analyses of archaeal XPD homologs revealing a doughnut-like folding whereby two RecA-like domains, an arch-like domain and an iron-sulfur cluster (FeS), build a deep channel and central hole that can accommodate single-stranded DNA [29–32]. These structural studies imply that DNA unwinding occurs by threading single DNA strands through this channel and the adjacent hole, thus bringing XPD in a strategic position to get into close contact with base lesions. Here, we demonstrate that the XPD helicase detects damaged substrates by a molecular interplay with such offending bases immediately before they enter the central protein pore, thereby revealing for the first time the mechanism of DNA lesion verification in a multilayered damage recognition process.

Results

NER Activity of XPD Mutants

The purpose of this study was to introduce amino acid changes by which XPD retains its ATPase and helicase activity

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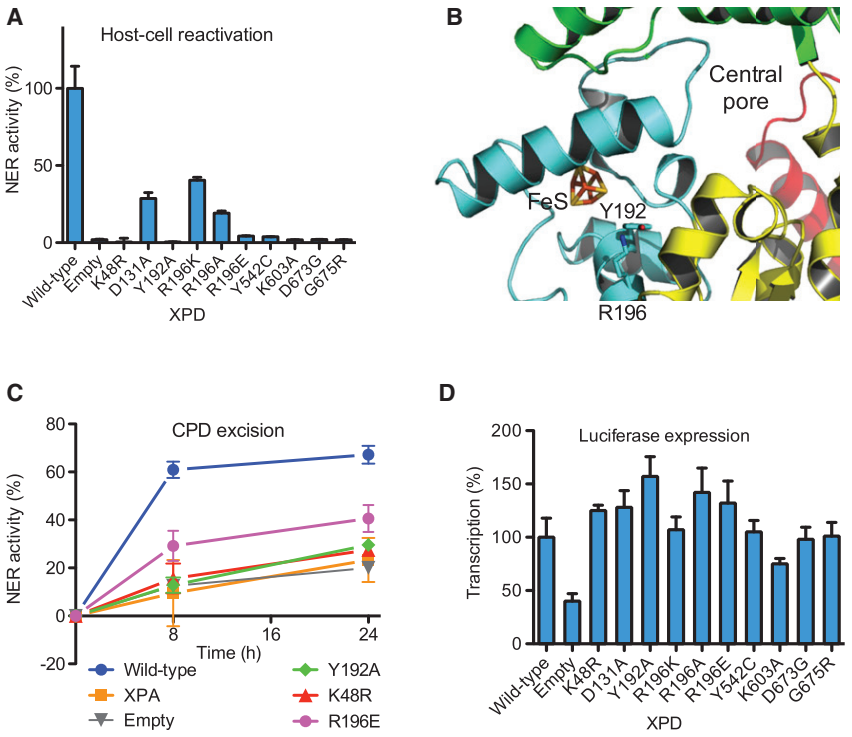

Figure 1. Generation of New XPD Mutants Targeting Evolutionarily Conserved Residues near the Central Protein Pore

(A) Screening of NER activity by host-cell reactivation assay in XP-D fibroblasts. NER capacity (indicated as percentage of wild-type control) is determined by the ratio of *Photinus* and *Renilla* luciferase production from UV-irradiated (pGL3) and undamaged reporter plasmid (pRL-TK), respectively (n = 6, ±SD). "Empty" indicates XP-D cells transfected with control expression vector coding only for GFP.

(B) Structural model illustrating the localization of Y192 and R196, defining a lesion recognition pocket in close proximity to the central pore of XPD protein (PDB accession code 4A15). FeS, iron-sulfur cluster. See Figure S1B for the full structure of archaeal XPD homologs [29–32].

(C) Stimulation of CPD excision in UV-irradiated (10 J/m^2) CHO cells by wild-type XPD and selected mutants (n = 5, ±SD). CPD levels were measured by immunoassay analysis of genomic DNA.

(D) Stimulation of transcription in XP-D cells. The transcriptional activity (indicated as percentage of wild-type control) is determined by the amount of *Photinus* luciferase production from the undamaged reporter plasmid pGL3 (n = 6, ±SD).

but loses the ability to discriminate between undamaged and damaged DNA during the unwinding reaction. Therefore, evolutionarily conserved residues in the proximity of the central hole, but not belonging to the distinctive signature motifs characteristic of DNA and RNA helicases [33] (see Figure S1A available online), were selected for site-directed mutagenesis. To test for functional implications, we performed a host-cell reactivation assay that determines the cellular NER capacity using a dual luciferase reporter system [34, 35]. NER-deficient XP-D fibroblasts were transfected with three constructs: a UV-irradiated (254 nm wavelength, 1,000 J/m^2) reporter plasmid containing the *Photinus* luciferase sequence, an undamaged control encoding the *Renilla* luciferase, and an expression vector for XPD fused to green fluorescent protein (GFP). Upon transfection, the NER machinery is needed to remove UV lesions from the irradiated reporter plasmid and allow for *Photinus* luciferase production. Finally, NER efficiency is monitored by measuring luciferase levels in cell lysates and recorded as the ratio of *Photinus* to *Renilla* activity.

The tested XP-D fibroblasts carry heterozygote mutations where one allele codes for an R683W substitution and the second allele yields a deletion of codons 36–61. Thus, XP-D cells that were transfected to express XPD-GFP containing the K48R mutation (located in helicase motif I) display only a background NER capacity equal to that detected with the empty GFP control (Figure 1A). This finding is consistent with a low residual NER function in XP-D cells and the inability of the enzymatically inactive K48R mutant to complement this deficiency [36]. A full repair activity is reestablished by expression of XPD-GFP displaying the wild-type sequence. Instead, the newly generated XPD mutations at codons Y192 and R196, although located outside the canonical helicase motifs, confer a NER defect as demonstrated by the low expression of *Photinus* luciferase. In the case of codon 196, a charge inversion by replacement with glutamic acid (R196E) caused a stronger reduction of NER activity than substitutions with the nonpolar alanine (R196A) or the positively charged lysine (R196K). For comparison, we introduced amino acid changes at positions D131 (mapping to a region between helicase motifs I and II) and K603 (mapping to helicase motif V), thereby obtaining different degrees of NER inhibition (Figure 1A). We also tested the known patient mutations Y542C, D673G, and G675R. Consistent with previous studies revealing that these pathological substitutions affect ATPase and DNA helicase functions [29, 30], the corresponding XPD derivatives are unable to correct the NER deficiency of XP-D cells.

To summarize, an initial screen by host-cell reactivation assay indicated that the Y192A and R196E mutations, buried in the deep DNA-binding channel in proximity to the central protein pore (Figures 1B and S1B), lead to diminished DNA repair. This conclusion was confirmed by monitoring the excision of UV damage from the genome of Chinese hamster ovary (CHO) cells with an immunoassay using antibodies against CPDs, the main type of bulky UV lesion. On their own, these rodent cells are characterized by slow repair of CPDs because they lack the damaged DNA-binding protein DDB2 that enhances CPD recognition [37]. However, we observed that wild-type XPD-GFP stimulates the excision of CPDs compared to mock transfections with empty GFP vectors (Figure 1C), implying that the ectopic overexpression of human XPD compensates, under the condition of this study, for the UV lesion recognition deficiency of CHO cells. This novel observation was exploited to assess the NER capacity of different XPD constructs, confirming that the enzymatically inactive K48R mutant fails to support CPD excision. Similarly, the Y192A substitution generates an XPD mutant that is unable to stimulate repair activity, and also the R196E change resulted in less CPD excision. During an initial 8 hr incubation, the observed DNA repair stimulation was reduced by as much as 99.4% and 65.5% with the Y192A and R196E mutations, respectively, in comparison to the wild-type reference (Figure 1C).

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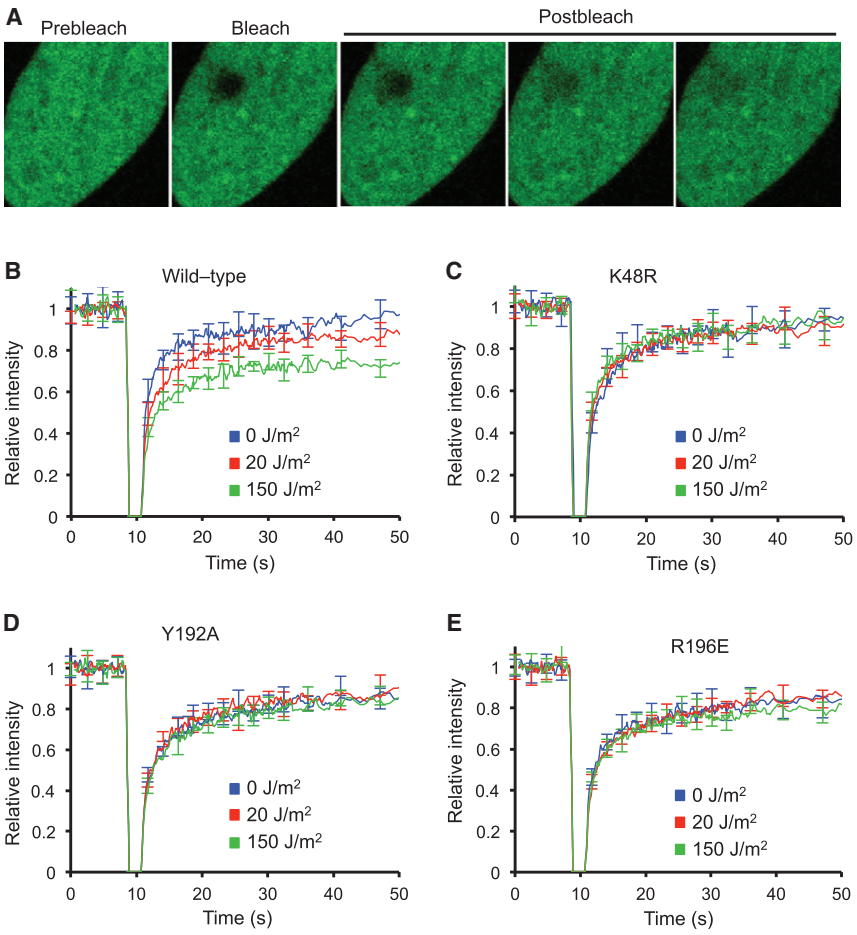

Figure 2. Recognition of UV Lesions in the Nuclei of Living Cells

(A) Typical series of images obtained when XPD protein dynamics were analyzed by FRAP. The postbleaching fluorescence recovery results from the movement of fluorescent proteins within the nuclear compartment.

(B) Delayed nuclear dynamics of wild-type XPD-GFP in UV-irradiated cells due to the recognition of UV lesions (n = 15, ±SEM).

(C–E) Defective recognition of UV lesions by mutated XPD carrying the indicated amino acid substitutions (n = 15, ±SEM).

The concurrent transcriptional activity has been determined to rule out the possibility that the tested amino acid substitutions derange DNA repair by interfering grossly with XPD protein folding or subunit assembly in the TFIIH complex. For that purpose, we made use of the observation that transfection of XP-D fibroblasts with the wild-type XPD-GFP construct stimulates luciferase expression from an undamaged reporter plasmid compared to controls carrying the empty GFP vector (Figure 1D). This effect is likely due to a subtle transcriptional defect of XP-D cells conferred by the R683W mutation in the remaining functional XPD allele, presumably disturbing the overall TFIIH structure [38]. The enhancement of reporter gene expression observed in the presence of wild-type XPD provides an opportunity to gauge the transcriptional activity of XPD mutants. Interestingly, at least the same stimulation of reporter gene expression is detected with all XPD constructs except K603A (Figure 1D), indicating that, despite their functional defect in repair, these mutants still display sufficient structural integrity to support transcription.

Nuclear Dynamics of XPD Mutants

The next question was whether the XPD mutations K48R, Y192A, and R196E diminish DNA repair by interfering with the proper interaction of XPD with UV lesions. To that end, we expressed XPD-GFP fusions in CHO cells and determined their overall nuclear mobility by fluorescence recovery after photobleaching (FRAP). In this real-time technique, a 4 μm^2 nuclear area was photobleached with a laser (488 nm wavelength) to locally suppress the fluorescence signal. The subsequent recovery of green fluorescence, resulting from the progressive movement of XPD-GFP molecules into the bleached nuclear area, was recorded over time (Figure 2A). Upon UV irradiation (254 nm), the fluorescence recovery of wild-type XPD is reduced in a dose-dependent manner, reflecting restrained protein mobility due to interactions with DNA damage (Figure 2B). The elevated UV dose of 150 J/m^2 was included to confront the NER system with a high lesion density. However, the nuclear dynamics of the mutant XPD proteins K48R, Y192A, and R196E were not altered by any of these UV treatments (Figures 2C–2E), indicating that, unlike the wild-type control, their movement remained unaffected.

A possible caveat of the protein dynamics studies of Figure 2 is that the mobility of XPD mutants was generally lower than wild-type in the absence of DNA damage such that these reduced protein dynamics might obscure a transient recruitment to damaged sites. As a consequence, we conducted further imaging experiments to analyze in detail the interaction of each mutant with DNA lesions in the chromatin context. For that purpose, CHO cells transfected with the different XPD-GFP constructs were UV irradiated through the pores of polycarbonate filters, followed by visualization of the resulting spots of UV damage by immunostaining with an antibody against CPDs. It was expected from previous reports using the inactivating K48R substitution [39, 40] that the tested XPD mutants, as long as they associate with the TFIIH complex, would be recruited to the vicinity of UV-damaged sites. Indeed, all tested XPD-GFP constructs, wild-type or mutant, accumulated at the sites of CPD formation, confirming that the fusion proteins are incorporated into TFIIH complexes and therefore are recruited to damaged DNA sites (Figure 3A). However, the quantitative comparison of local fluorescence intensity over the surrounding nuclear background, in cells expressing equal overall levels of XPD-GFP fusions, revealed an intriguing difference in the degree of damage-specific protein accumulation. Wild-type XPD reached the highest fluorescence intensity at lesion sites and hence a stronger UV-dependent accumulation than the mutants K48R, Y192A, and R196E, which relocated to UV sites less efficiently (Figure 3B).

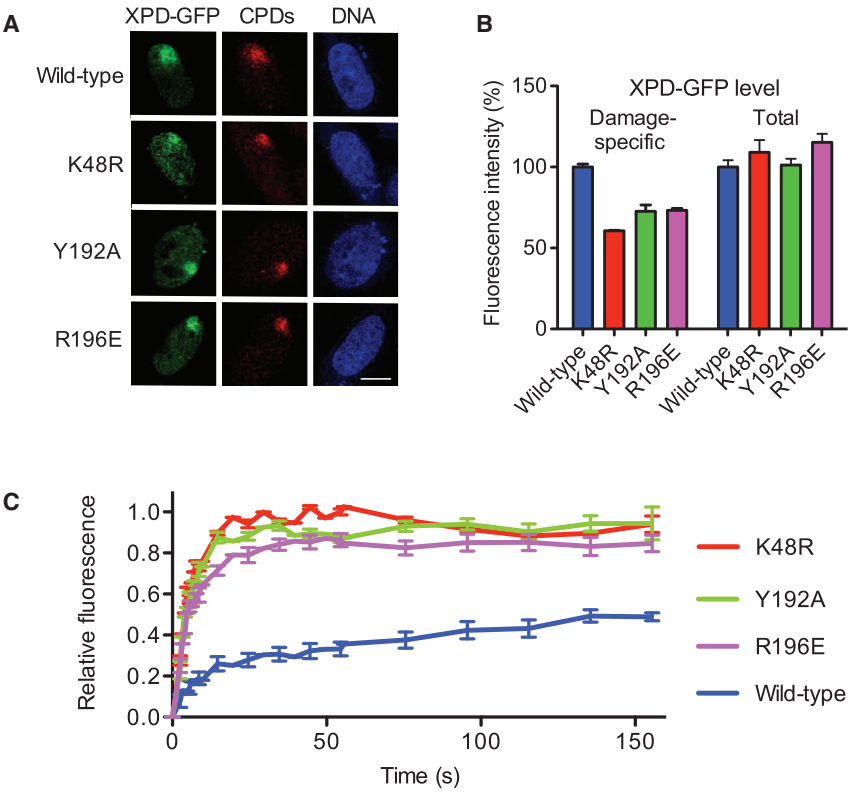

Figure 3. Stability of DNA Damage Recognition Intermediates in the Nuclei of Living Cells

(A) Formation of spots of UV lesions (CPDs) by irradiation through micropore filters and accumulation of XPD-GFP at the sites of DNA damage. The DNA is visualized by staining with Hoechst reagent.

(B) Quantification of XPD-GFP fusions that accumulate at spots of UV lesions in comparison to the overall expression (as percentage of wild-type control; n = 30, ±SEM).

(C) Residence time of XPD-GFP fusions at UV lesion spots determined by FRAP-LD (n = 15, ±SEM).

(D) UV-dependent sequestration of XPD-GFP in chromatin visualized by MNase digestion. "Free" indicates fraction of non-chromatin-bound XPD-GFP removed by salt extraction (0.3 M NaCl) before MNase treatment. TBP, TATA-binding protein used as a marker of chromatin-bound proteins susceptible to MNase solubilization.

See also Figure S2.

The underlying cause of this difference in protein redistribution was examined by FRAP on local damage (FRAP-LD) analyses. Briefly, the fluorescence of individual green spots of XPD-GFP accumulation at lesion sites was photobleached to reduce the local intensity to that of the nuclear background. The fluorescence recovery due to an exchange of bleached XPD-GFP molecules on lesion sites with nonbleached counterparts from surrounding undamaged regions was then monitored over time, thereby providing real-time kinetics of the interplay between XPD protein and DNA lesions. The resulting fluorescence recovery curves revealed that a large proportion of wild-type XPD persists at lesion sites (Figure 3C). In contrast, as demonstrated earlier [40], the K48R active-site mutant was released rapidly and completely from UV lesions. In this study, similarly fast dissociations were detected with the new mutants Y192A and K196E (Figure 3C). Representative FRAP-LD images generated with wild-type and mutant XPD are shown in Figure S2. The diverging FRAP-LD recovery curves indicate that, like K48R, the Y192A and R196E substitutions result in reduced immobilization at DNA damage. We therefore concluded that residues Y192 and R196 are required for the formation of stable lesion recognition intermediates.

Prompted by these findings, we used a standard chromatin digestion assay [41] to visualize the fraction of XPD-GFP that, in UV-irradiated CHO cells, becomes closely associated with damaged DNA. First, the free XPD-GFP molecules not bound to chromatin were removed by salt (0.3 M NaCl) extraction. Second, the remaining XPD-GFP moieties interacting tightly with chromatin were released by chromatin solubilization through micrococcal nuclease (MNase) digestion. As shown in Figure 3D, this treatment demonstrated that part of wild-type XPD is sequestered in chromatin upon UV irradiation. However, none of the mutant proteins formed a comparable association with damaged chromatin, thus confirming their inability to induce stable recognition intermediates. To rule out that the 0.3 M NaCl washing step might inadvertently remove XPD mutants from DNA, we repeated the same experiment using 0.05 and 0.15 M NaCl, but in both cases the mutant proteins remained unable to associate with damaged chromatin despite the lower salt concentration.

DNA-Binding Capacity of XPD Mutants In Vitro

Next, the role of residues Y192 and R196 in damage recognition was tested in the framework of a monomeric XPD homolog from *Ferroplasma acidarmanus* (FaXPD) that is closely related in sequence and, unlike other frequently used archaeal homologs, active at moderate temperature, thus providing an excellent model enzyme for the human counterpart [42, 43]. The archaeal residues K37 (corresponding to K48 in human XPD), Y171 (Y192 in human XPD), R175 (R196 in human XPD), and K523 (K603 in human XPD; see Figure S1A) were mutated, and the resulting FaXPD derivatives, fused to an N-terminal His_6 tag, were produced in *Escherichia coli* and purified to homogeneity (Figure S3A). To facilitate comparisons, the human codon numbering has been adopted for the corresponding FaXPD residues.

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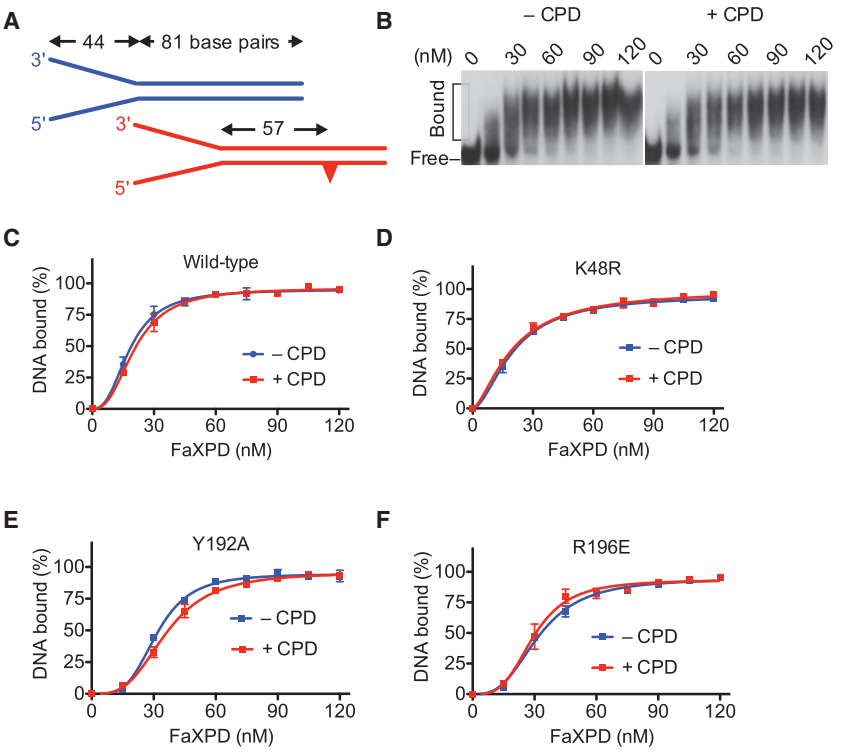

Figure 4. Binding of FaXPD Proteins to Undamaged and Damaged DNA Substrates

(A) Scheme of forked DNA substrates illustrating the position of a single CPD.

(B) Representative electrophoretic mobility shift assay demonstrating that the association of FaXPD with DNA substrate (5 nM) is not affected by the presence of a single CPD.

(C) Quantification of mobility shift assays carried out with wild-type FaXPD (n = 5, ±SD).

(D–F) Quantification of mobility shift assays performed with mutant FaXPD proteins containing the indicated amino acid substitutions (n = 5, ±SD). See also Figure S3.

We compared the DNA-binding activity of wild-type FaXPD and the K603A mutant, where an amino acid substitution in helicase motif V was expected to interfere with substrate interactions. Electrophoretic mobility shift assays were carried out using forked DNA duplexes consisting of 81 bp segments flanked by single-stranded arms of 44 nucleotides (Figure S3B). These DNA-binding assays revealed that wild-type FaXPD readily forms nucleoprotein complexes and that the resulting associations saturate at protein concentrations around 60 nM (Figure S3C). In comparison, the K603A mutant exhibits a markedly reduced DNA-binding activity at low protein concentrations, and accordingly, higher amounts of mutant FaXPD are needed to achieve binding saturation (Figure S3D).

Further mobility shift assays using oligonucleotides containing a lesion demonstrated that a single CPD in the duplex region of forked DNA (Figure 4A) does not interfere with the association of FaXPD with its substrate (Figures 4B and 4C). Similarly, no differences were observed between the DNA binding of the K48R variant in the presence or absence of a CPD (Figure 4D). Conversely, the mutations Y192A and R196E reduce the binding of FaXPD to the forked DNA substrate, but only at low protein concentrations (Figures 4E and 4F). Importantly, with both mutants, protein-DNA interactions were saturated at a FaXPD concentration around 60 nM, and in all cases, the presence of a CPD did not interfere with their DNA-binding properties. The resulting equilibrium dissociation constants (K_D), reflecting the affinity for forked DNA, were 20 nM (for wild-type XPD and the K48R mutant), 31 nM (for R196E), 34 nM (for Y192A), and 45 nM for the K603A mutant, the lowest affinity detected.

Failure of XPD Mutants to Sense Damage during DNA Unwinding

The same forked substrates were used to test the consequences of a collision with base lesions during DNA unwinding. The DNA helicase activity of wild-type FaXPD is severely inhibited when encountering a single CPD located in the 5′–3′ translocated strand [40] (Figure 5A). By extending this analysis to the Y192A and R196E mutants, we noted that, at the FaXPD protein concentration of 60 nM or higher (the DNA-saturating range in the binding assays of Figure 4), strand displacement from the undamaged duplex was only marginally reduced compared to the wild-type control. The outstanding finding was that, independently of protein concentration, the DNA helicase activity of the Y192A and R196E mutants is not affected by a CPD lesion (Figures 5B and 5C). Thus, both mutants are able to progress to the substrate termini and achieve complete strand separation after overriding the template lesion, indicating that residues Y192 and R196 are necessary to sense DNA damage. The other tested FaXPD derivatives (K48R and K603A) were devoid of DNA-unwinding activity (data not shown), consistent with the fact that residues K48 and K603 are located within the evolutionarily conserved helicase motifs.

We also tested the corresponding ATPase activity in the presence or absence of a CPD in forked DNA substrates. Neither the wild-type FaXPD enzyme nor the Y192A and R196E mutants were affected by the presence of a CPD lesion with regard to their ability to promote ATP hydrolysis (Figure S4). On the other hand, this ATPase activity was completely abolished with the K48R and K603 mutants (data not shown), which is in line with the concomitantly missing helicase function.

Failure of XPD Mutants to Form a Stable Verification Intermediate

We previously established competition and nuclease protection assays to demonstrate that FaXPD generates a stable complex with DNA after running into a CPD lesion [40]. For example, preincubation of wild-type FaXPD with a radiolabeled 51-mer DNA oligonucleotide generates nucleoprotein complexes that, in the presence of ATP, disappear after the addition of a 50-fold molar excess of unlabeled 51-mer (Figure 6A). In these reactions, which contain ATP and undamaged substrate, FaXPD moves to the 3′ end, where it is released from the labeled oligonucleotide and reassociates preferentially with nonlabeled competitors. Instead, in reactions containing no ATP or supplemented with the nonhydrolyzable

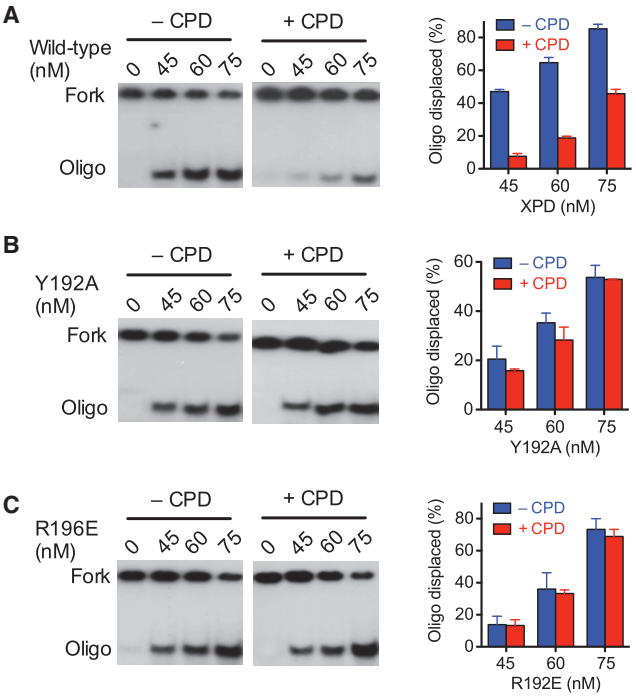

Figure 5. Differential Unwinding of Damaged DNA Duplexes

(A) A single CPD inhibits the DNA helicase activity exerted by wild-type FaXPD on forked substrates. The panel on the right shows the quantification of five independent experiments (±SD).

(B) The same CPD substrate fails to inhibit DNA unwinding by the Y192A mutant (n = 5, ±SD).

(C) Similarly, the CPD is unable to inhibit DNA unwinding by the R196E mutant (n = 5, ±SD).

See also Figure S4.

analog ATPγS, the FaXPD enzyme cannot move to the 3′ terminus and hence fails to dissociate giving rise to stable complexes refractory to competition. Similarly, the presence of a single CPD lesion within the radiolabeled 51-mer oligonucleotide results in the formation of stable nucleoprotein associations between FaXPD and DNA even when the reaction mixtures contain ATP, indicating that the helicase is stalled at lesion sites (Figure 6A). The enzymatically inactive K48R and K603A mutants yield always stable complexes in these competition assays because they are unable to undergo ATP hydrolysis-dependent translocation toward the 3′ end of DNA substrates (Figures 6B and 6C).

A completely different outcome was detected when the Y192A and R196E mutants were probed in competition assays. Like wild-type XPD, both mutants readily dissociate from the radiolabeled oligonucleotide in the absence of DNA lesions if the reactions are supplemented with ATP. However, the Y192A and R196E derivatives are unable to form the expected stable nucleoprotein complex following ATP-driven collisions with a CPD site. These incubations yield an incomplete electrophoretic mobility shift, whereby part of the radiolabeled oligonucleotides remains in the protein-bound state but a considerable proportion of mutant enzymes is released from the DNA substrate (Figures 6D and 6E). The faster migrating bands, indicated by an asterisk, represent partially dissociated XPD-DNA complexes. Thus, the Y192A and R196R substitutions generate XPD derivatives that interact less efficiently with DNA damage than the wild-type control.

These findings obtained in competition assays were confirmed by testing the ability of FaXPD mutants to protect DNA from digestion by T4 endonuclease V (T4 endo V), which specifically recognizes and cleaves DNA at CPD positions [44]. After a 15 min preincubation of forked DNA substrate with FaXPD, the unwinding products were probed by the addition of T4 endo V. A CPD-dependent cleavage is indicative of protein-free DNA regions, whereas reduced cleavage would demonstrate occlusion of the CPD site by a close interaction of XPD with DNA lesions. If, as shown in the denaturing gel of Figure 6F, the preceding helicase reaction was performed with the inactive mutants K48R and K603A, which are unable to translocate along DNA, the CPD site in forked substrates remained protein free and accessible to cleavage by T4 endo V. Instead, preincubation with wild-type FaXPD restricted the cleavage by T4 endo V, indicating that the enzyme moves to the CPD site and forms intimate contacts with the lesion. Preincubation with the Y192A and R196E mutants failed to inhibit the digestion by T4 endo V, consistent with a less efficient masking of CPDs compared to the reactions with wild-type FaXPD. These results confirm that residues Y192 and R196 are necessary to sense DNA damage during the tracking movement of XPD and thereby generate a stable recognition intermediate.

Discussion

This report identifies critical amino acids by which XPD discriminates between undamaged and damaged DNA substrates, and by replacement of the respective side chains, we ultimately prove that this DNA helicase subunit of TFIIH serves as a general damage verifier in the NER pathway. We also demonstrate a previously postulated [25, 31] but thus far unproven new mode of DNA quality control involving the narrow protein pore of a nucleic acid-scanning enzyme.

Spontaneously formed *XPD* mutations in human patients, which give rise to XP, combined XP/CS, or TTD disorders, either cause a loss of DNA helicase activity or destabilize interactions with other TFIIH subunits [14, 29–31, 38, 45]. Here, we took a novel approach by targeting for site-directed mutagenesis a critical region of XPD where its DNA-binding channel reaches a narrow protein pore, but without replacing amino acids directly involved in ATPase and DNA helicase activity. Utilizing this strategy, we obtained two XPD mutants (Y192A and R196E) that retain the ability to unwind double-stranded DNA, although they fail to sense bulky base lesions during their ATP-driven scanning movement along nucleic acid lattices. Moreover, in the absence of the Y192 and R196 side chains, XPD shows a reduced ability to form stable DNA damage recognition intermediates. We conclude from these biochemical findings that residues Y192 and R196 delineate a sensor pocket, linked to the central protein pore, which is dedicated to the recognition of base lesions. This sensor pocket lies in a region that had been identified, using the archaeal homolog from *Thermoplasma acidophilum*, as a hot spot for interactions with DNA substrates [32, 46]. In support of our findings, Kuper et al. [32] observed previously that the *T. acidophilum* Y166A mutant, which is considered equivalent to the Y192A derivative of this report, also retains DNA helicase activity, although to a lesser degree than the human Y192A mutant. Our study is the first to characterize the consequences of this Y192A mutation, and the R196E substitution, in the physiologic chromatin context of living cells, thus demonstrating that the trapping of bulky base lesions by the newly identified

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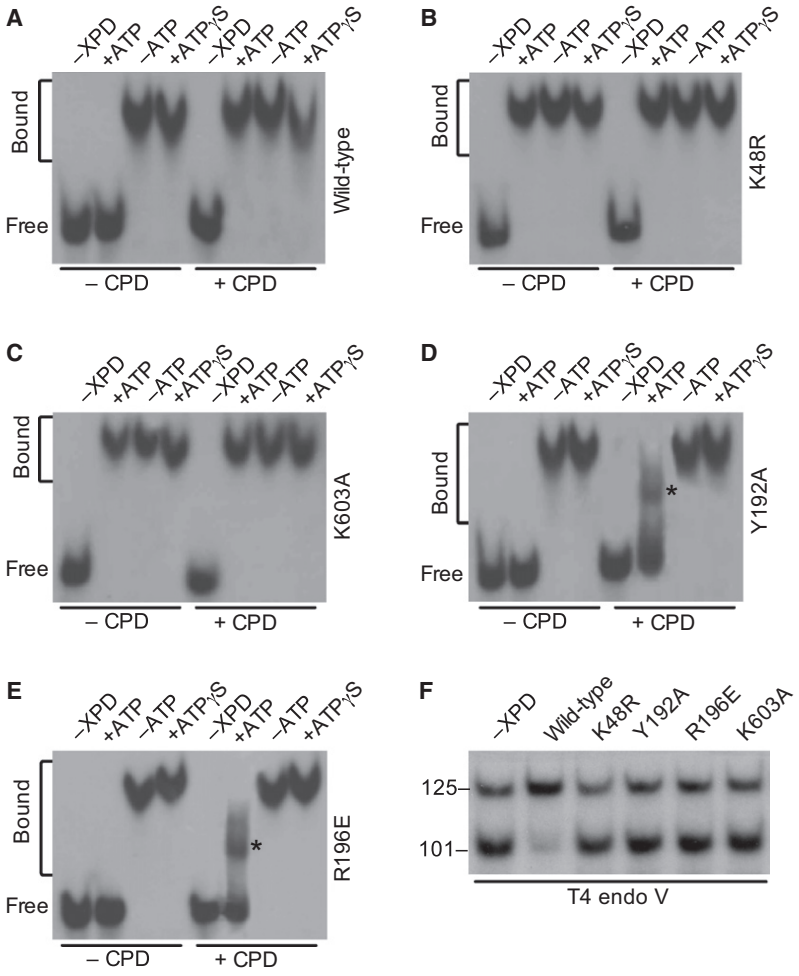

Figure 6. Immobilization of FaXPD at DNA Lesions

(A) Competition assay with wild-type FaXPD. In the presence of ATP (3 mM), the enzyme (60 nM) rapidly dissociates from the undamaged oligonucleotide (5 nM), but not from the damaged counterpart containing a single CPD. –XPD, control reactions without the addition of FaXPD; ATPγS, reactions containing nonhydrolyzable ATP analog.

(B and C) Competition assays with the enzymatically inactive K48R and K603A mutants.

(D and E) Competition assays with the DNA damage-insensitive Y192A and R196E mutants. Asterisks indicate partially dissociated XPD-DNA complexes.

(F) Protection assay with T4 endo V showing that wild-type FaXPD, but not the mutant enzymes, forms a demarcation complex at the CPD lesion site in forked DNA substrates.

sensor pocket (involving both Y192 and R196) is indeed a strict requirement for damage excision. That XPD depends on a dedicated recognition pocket, in addition to the protein pore, to detect base lesions is in line with a previous report showing that this helicase overcomes even large obstacles, like tightly bound single-stranded DNA-binding proteins, during its ATP-driven translocation [42].

The newly discovered DNA damage-sensing function of XPD bears on the finding that XPC, which is the upstream initiator of global genome NER activity, detects damage-induced destabilization of the double helix rather than recognizing abnormally modified bases [8, 35, 47]. This entirely indirect mode of repair initiation implies that the NER machinery must display a downstream recognition subunit that verifies base lesions to assure the presence of chemical modifications before carrying out the excision reaction. The critical importance of this verification mechanism is emphasized by the finding that XPC also binds extensively to undamaged DNA, indicating that, instead of being responsible for lesion recognition, it provides an essential but rather unspecific "matchmaker" for the loading of downstream NER factors onto the DNA double helix [7, 19–24, 28, 48]. In view of these interactions of XPC taking place on undamaged DNA, i.e., in the absence of chemical modifications, it is necessary to protect the native double helix, which is present in vast excess, from futile excision cycles. Conversely, during transcription-coupled repair, the temporarily stalled RNA polymerase II is removed from the template strand to expose the substrate duplex to the activity of TFIIH and further downstream NER factors [25–28, 49]. Therefore, an attractive feature of lesion verification, mediated by the tracking movement of XPD, is that in both global genome and transcription-coupled repair, this helicase ensures the correct timing and spacing of DNA unwinding, thereby providing a central decision point in the NER reaction. With this verification mechanism, a stably unwound DNA intermediate leading to damage excision is only formed by damage-specific stalling of XPD, in a way that strand separation by TFIIH, possibly in conjunction with additional factors like XPA [28], is strictly focused on lesion sites. In contrast, native regions of the double helix, which fail to stop the XPD helicase, are bypassed by dynamically translocating TFIIH complexes without being presented to the NER machinery.

Experimental Procedures

Materials

Simian virus 40-transformed XP-D fibroblasts (GM08207) were from the Coriell Institute for Medical Research (Camden, NY). The expression vector for XPD-GFP was kindly provided by W. Vermeulen (Erasmus Medical Center, Rotterdam, the Netherlands). The pGL3 and pRL-TK vectors expressing *Photinus* and *Renilla* luciferase, respectively, were from Promega.

Cell Culture

Culture media and supplements were from Invitrogen. XP-D cells were grown using Dulbecco's modified Eagle's medium, whereas V79 CHO cells were cultured using F-12 nutrient mixture in a humidified incubator at 37°C and 5% CO_2. These media were supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin.

DNA Repair Assays

Host-cell reactivation assays [35], CPD excision assays [24], and the immunochemical analysis of spots of DNA damage and repair, generated by UV irradiation through micropore filters, were carried out as outlined in Supplemental Experimental Procedures.

Image Analysis

Fluorescence measurements at UV radiation spots were performed through a 63× oil immersion objective with a numeric aperture of 1.4 (EC Plan-Neofluar, Zeiss) using an Ar^+ source (488 nm). The average fluorescence

intensities were assessed in the area of CPD formation and normalized against the background signal in a neighboring nonirradiated area of identical size. The background-corrected values are given as a percentage of the wild-type XPD-GFP signal. In addition, the overall green fluorescence level in the nuclei of transfected cells was analyzed, and only cells with comparably low XPD protein expression were used for quantifications.

Live-Cell Determination of Protein Dynamics

FRAP was performed on a Leica TCS SP5 confocal microscope, operated by Application Suite 2.6.3.8173, and equipped with an Ar^+ laser and a 63× oil immersion lens (numeric aperture 1.4). Protein dynamics assays were performed in a controlled environment (37°C, 5% CO_2). Briefly, transfected CHO cells were grown on 18 mm glass coverslips. A region of interest (ROI) of 4 mm^2 was photobleached for 20 iterations at 75% laser intensity. Then, fluorescence recovery within the ROI was monitored 200 times every 115 ms followed by 30 frames of 250 ms and 20 frames of 500 ms. The results were adjusted for overall bleaching by correction with a reference ROI of identical size at each time point. Finally, the first fluorescence measurement after bleaching was set to zero and the following data were plotted as a function of time [22, 50].

FRAP on local damage (FRAP-LD) was performed using a 40× oil immersion lens (numeric aperture 1.4). In transfected CHO cells, ROIs corresponding to sites of XPD-GFP accumulation were defined 30–45 min after the induction of repair spots by irradiation through polycarbonate filters. Each ROI was photobleached at 75% laser intensity until the fluorescence reached a level equivalent to that outside the UV spot. Subsequently, the fluorescence recovery was monitored ten times using 700 ms intervals followed by eight frames of 5 s and five frames of 20 s. Concomitantly, a reference ROI of the same size was measured to correct for overall bleaching. In the data display, the first fluorescence measurement after photobleaching is set to zero, and all following time points are normalized to the prebleaching level [40, 50].

Biochemical Methods

The chromatin binding assay and characterization of purified FaXPD proteins were carried out as described previously [24, 40] and outlined in Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.12.032.

Acknowledgments

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Supplemental Information

DNA Quality Control by a Lesion Sensor Pocket of the Xeroderma Pigmentosum Group D Helicase Subunit of TFIIH

Nadine Mathieu, Nina Kaczmarek, Peter Rüthemann, Andreas Luch, and Hanspeter Naegeli

Supplemental Inventory

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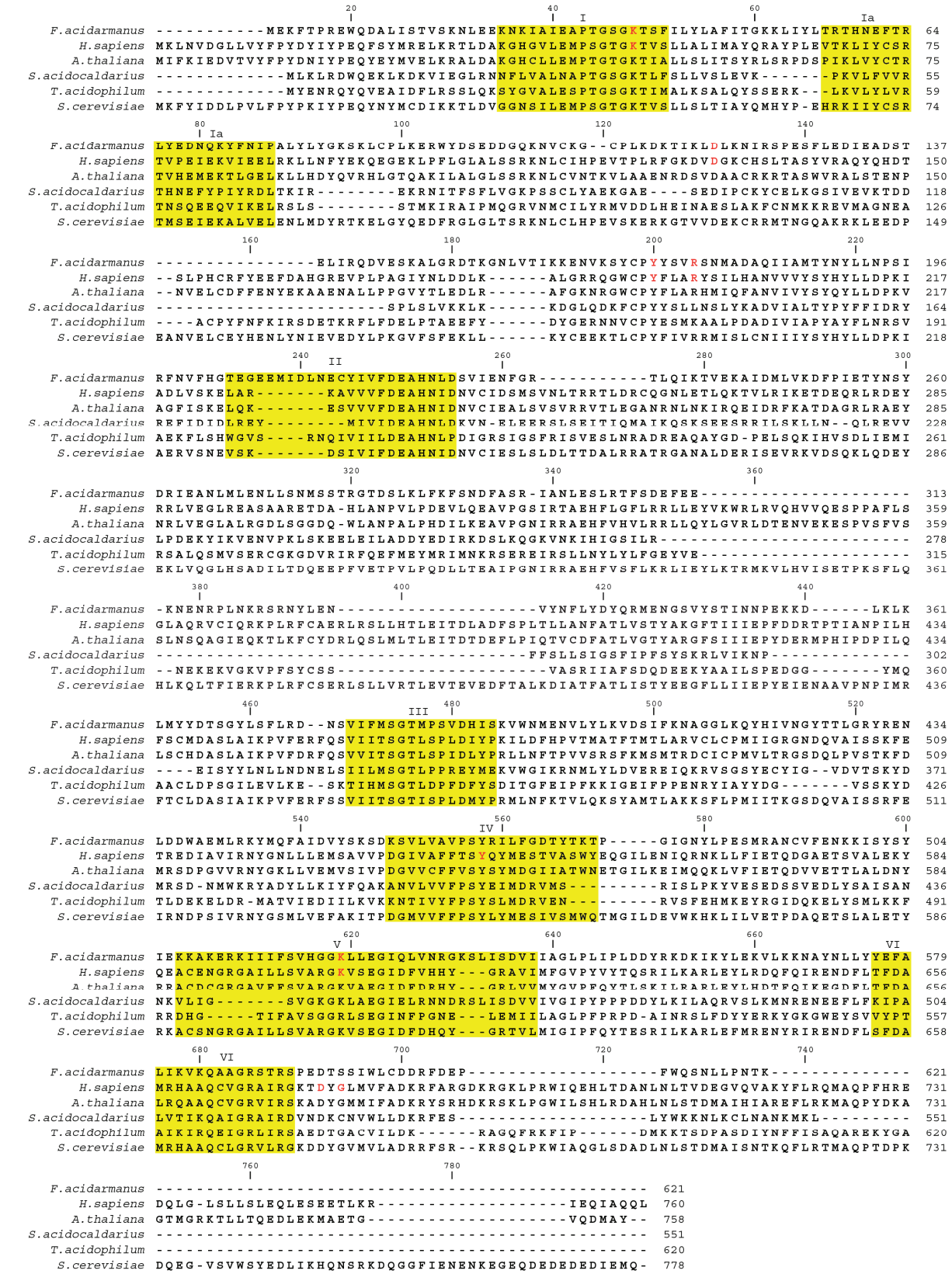
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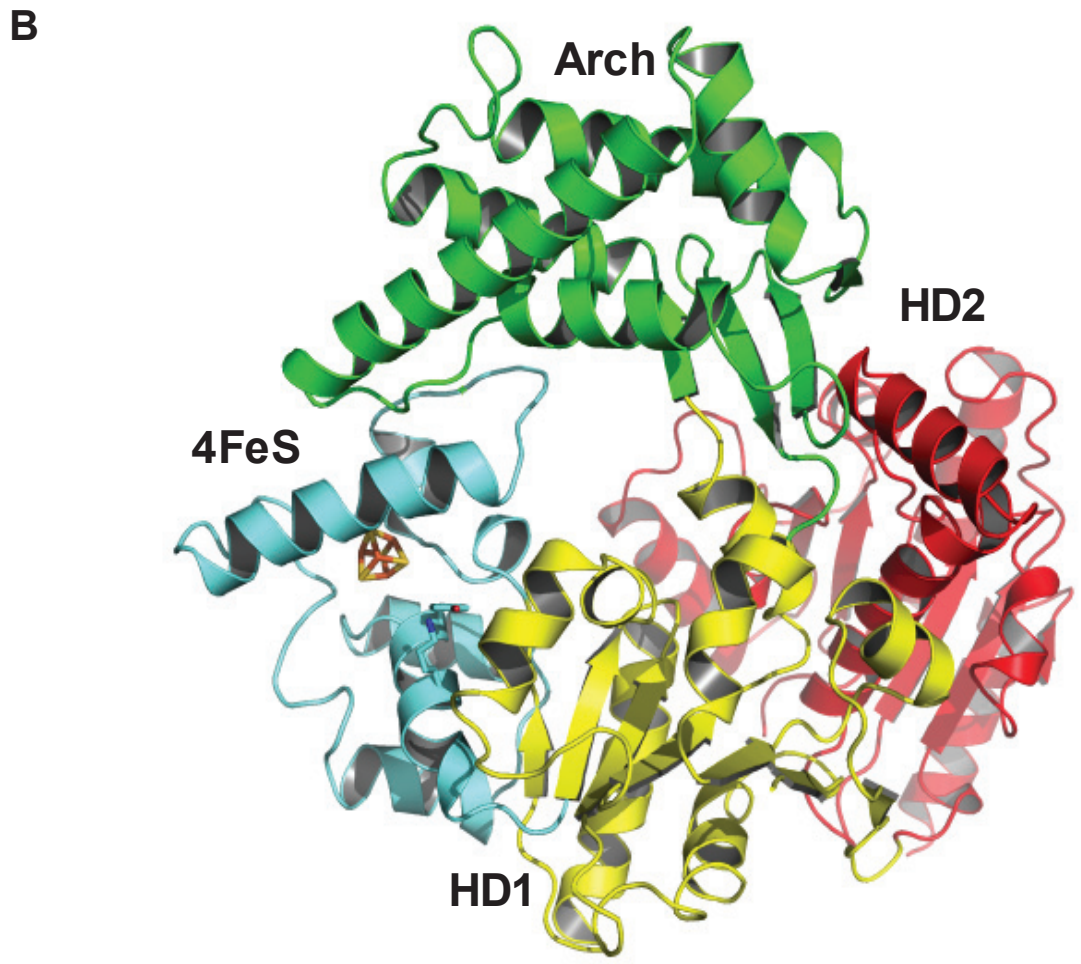

Figure S1. Overall Structure of XPD Protein

(A) The XPD sequences from *Ferroplasma acidarmanus*, *Thermoplasma acidophilum*, *Sulfolobus acidocaldarius*, *Homo sapiens*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae* have been aligned (ClustalW algorithm using the CLC Sequence Viewer 6.7.1) to illustrate the relative position of evolutionary conserved helicase signature motifs (highlighted with yellow boxes) [1] and residues subjected to substitution (in red color). The motifs I and II are also referred to as Walker A and B, respectively. We hypothesized that replacements of Y192 and R196 (in the human sequence) would abolish DNA damage recognition by XPD without compromising DNA helicase activity.

(B) Domain structure of XPD from *T. acidophilum* [2]. Arch, arch-like domain, HD1 and HD2, RecA-like helicase domains 1 and 2; 4FeS, iron sulfur cluster (PDB accession code 4a15).

3.2 NER Damage Sensors Turnover by p97 in the Chromatin

Section 3.2 constitute an article (Puumalainen *et al*, 2014) published 2014 in the journal *Nature Communications* and is entitled:

Chromatin retention of DNA damage sensors DDB2 and XPC through loss of p97 segregase causes genotoxicity

This paper describes the functional cooperation between p97 (Valosin-containing protein) and UV lesion recognition proteins DDB2 and XPC. *M. Puumalainen*, *D. Lessel, N. Kaczmarek, K. Bachmann* and *P. Rüthemann* showed that p97 is beneficial for efficient NER, since it removes polyubiquitinated DDB2 and XPC from UV lesions and allows unimpaired NER.

I designed, performed and analysed the following experiments in this study:

(1) 6-4PP lesion repair assay of p97 depleted human cells (**Fig. 5a**)

(2) CPD lesion repair assay of p97 depleted human cells (**Fig. 5b**)

(3) 6-4PP lesion repair assay after DDB2 overexpression (**Suppl. Fig 5d**)

(4) CPD lesion repair assay after DDB2 overexpression (**Suppl. Fig 5e**)

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Chromatin retention of DNA damage sensors DDB2 and XPC through loss of p97 segregase causes genotoxicity

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DNA damage recognition subunits such as DDB2 and XPC protect the human skin from ultraviolet (UV) light-induced genome instability and cancer, as demonstrated by the devastating inherited syndrome xeroderma pigmentosum. Here we show that the beneficial DNA repair response triggered by these two genome caretakers critically depends on a dynamic spatiotemporal regulation of their homeostasis. The prolonged retention of DDB2 and XPC in chromatin, because of a failure to readily remove both recognition subunits by the ubiquitin-dependent p97/VCP/Cdc48 segregase complex, leads to impaired DNA excision repair of UV lesions. Surprisingly, the ensuing chromosomal aberrations in p97-deficient cells are alleviated by a concomitant downregulation of DDB2 or XPC. Also, genome instability resulting from an excess of DDB2 persisting in UV-irradiated cells is prevented by concurrent p97 overexpression. Our findings demonstrate that DNA damage sensors and repair initiators acquire unexpected genotoxic properties if not controlled by timely extraction from chromatin.

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Exposure to the ultraviolet (UV) radiation of sunlight induces DNA lesions such as cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine–pyrimidone photoproducts (6-4PPs), which, if not repaired, lead to sun hypersensitivity, genome instability and skin cancer[1–4]. Excision of this highly mutagenic DNA injury occurs by the nucleotide excision repair (NER) pathway, which depending on the precise lesion location is triggered by two distinct DNA damage recognition routes. The transcription-coupled NER reaction is initiated by RNA polymerase II (POLII)-blocking lesions and, therefore, removes DNA damage exclusively from the transcribed strand of active genes[5,6]. In contrast, the global-genome NER reaction takes advantage of the lesion sensors DDB2 (for damaged DNA-binding 2) and XPC (for xeroderma pigmentosum group C) to recognize and remove DNA damage from both transcribed and non-transcribed templates across the whole genome[7,8]. Although XPC is the core sensor that detects a wide range of helix-distorting photoproducts and DNA adducts independently of the exact nature of the offending damage[7,9,10], DDB2 is an accessory NER subunit that specializes on the recognition of UV-induced CPDs and 6-4PPs[11–15]. In addition to interacting with XPC[16,17], DDB2 associates with DDB1 thereby forming a heterodimer, designated as UV-DDB, that provides a bridge for the recruitment of cullin 4A/B (Cul4A/B)-RING ubiquitin ligases[16,18–22]. Following UV radiation, the major source of genome instability in human skin, the UV-DDB and Cul4A/B ubiquitin ligase complexes move to DNA lesions and ubiquitinate various acceptor proteins including DDB2 and XPC. However, the role of this ubiquitination reaction and its link to the cellular DNA damage response remained elusive and, in particular, it was enigmatic why, by this process, UV radiation induces the degradation of most DDB2 subunits well before excision of the arising photoproducts is completed[23,24].

The ubiquitin-selective p97 segregase, also known as valosin-containing protein (VCP) or Cdc48 in yeast, is an ATP-driven molecular chaperone that drives the remodelling of ubiquitinated proteins to facilitate their degradation or recycling[25,26]. Chromatin-associated functions of p97 include extraction of the Aurora B kinase from mitotic chromosomes[27] or release of the alpha2 and L3MBTL1 repressors from DNA targets[28,29]. Previous reports that the p97 segregase is involved in the remodelling of transcription[30] or replication machineries stalling at UV lesions[31–36], prompted us to test whether DDB2 and XPC, once ubiquitinated after UV irradiation, may become susceptible to regulation by this multifunctional chaperone. We found that the p97 segregase recognizes ubiquitinated DDB2 and XPC, and that its recruitment to UV lesions, in addition to Cul4A ubiquitin ligase activity, requires the ubiquitin-binding adaptors Npl4, Ufd1 and UBXD7. Failure to remove ubiquitinated DDB2 or XPC, upon p97 depletion or expression of a segregase-inactive p97 mutant, causes retention of both DNA damage sensors in chromatin and impairs the NER reaction, thereby leading to chromosomal aberrations after exposure to UV light. This report thus reveals that the early DNA damage sensors DDB2 and XPC function as a double-edged sword, as they are essential to trigger a beneficial DNA repair activity but, if allowed to accumulate in damaged chromatin without control by the p97 segregase, may become detrimental to the genome.

Results

Ubiquitin-dependent recruitment of p97 to UV lesions. To monitor the recruitment of p97 complexes to UV lesions, spots of local damage were generated in the nuclei of human U2OS cells by UV radiation through the pores of polycarbonate filters. Endogenous p97 and a mildly overexpressed p97, tagged with a myc epitope (p97-myc), co-localized with CPDs (Fig. 1a), confirming that this segregase participates in the UV damage response. Time courses with endogenous p97 established the transient nature of this recruitment as all segregase complexes disappeared from the spots of UV damage formation within 24 h after irradiation (Supplementary Fig. 1a, b). Individual p97 subunits assemble to form hexamers that convert ATP hydrolysis into mechanical force by which target proteins are removed from cellular structures such as chromatin[25,26]. Therefore, the accumulation of p97 at CPD sites was markedly increased in the presence of an ATPase-inactive, dominant-negative mutant (p97 EQ) that still binds to ubiquitinated proteins but lacks segregase activity and, therefore, remains trapped on ubiquitinated substrates[37] (Fig. 1a,b).

Next to demonstrate that the p97 relocation to UV lesions is ubiquitin dependent, the nuclear compartment was depleted of its free ubiquitin pool by treatment with the proteasome inhibitor MG132 (ref. 38). The effective depletion of ubiquitin in the nuclear compartment is demonstrated, for example, by the missing ubiquitination of XPC following a standard UV insult (Supplementary Fig. 1c). This condition of nuclear ubiquitin depletion nearly abolished p97 EQ accumulation at CPD sites, confirming that protein ubiquitination is an essential signal for the p97 recruitment (Fig. 1c,d). Three different cullins (Cul3, Cul4A and Cul4B) are involved in K48-linked ubiquitination at UV damage[4,16,18,23]. Concomitant depletion of Cul4A and Cul4B by treatment with small interfering RNA (siRNA) caused a more severe reduction of p97 EQ relocation to CPDs than suppression of Cul3 (Fig. 1c,d; see Supplementary Fig. 1d for the efficiency of siRNA-mediated protein downregulation). A side-by-side comparison showed that Cul4A rather than Cul4B is primarily responsible for the p97 accumulation at CPDs (Supplementary Fig. 1e,f).

That p97 has a role in the processing of ubiquitinated substrates at UV lesions was supported by comparing the amounts of nuclear K48-linked ubiquitin (Fig. 1e). Depletion of p97 by siRNA treatment increased the level of ubiquitin chains not only in the nucleus overall but also at CPD spots. Figure 1f,g shows the quantifications of K48-linked ubiquitin levels in the whole nucleus and at CPD spots, the latter determined by measuring the ratio of fluorescence signals relative to the nuclear surroundings. In conjunction, these findings indicate that p97 promotes the removal of Cul4A-ubiquitinated substrates from the chromatin overall but also from UV lesion sites.

Recruitment of p97 segregase to UV lesions is DDB2 dependent. A further siRNA screen (see Supplementary Fig. 2 for the efficiency of protein depletion) showed that the recruitment of p97 to UV lesions is primarily dependent on DDB2, the damage sensor subunit of the UV-DDB heterodimer (Fig. 2a). To a lesser degree, p97 is also recruited to UV damage by XPC, but no effect on the p97 localization was detected following depletion of XPA, which acts downstream of the early sensors DDB2 and XPC in the NER pathway (Fig. 2a,b). We therefore hypothesized that DDB2 is a main p97 substrate.

That p97 interacts with a DDB2-containing protein complex was shown by co-immunoprecipitation studies using human embryonic kidney 293 (HEK293) cells mildly expressing the p97 EQ mutant, tagged with a Strep peptide, together with FLAG epitope-tagged DDB2. A pull-down of DDB2-FLAG with anti-FLAG beads (coated with antibodies against the FLAG peptide) and analysis of the resulting eluates by western blotting demonstrated that p97 and DDB2 reside in the same multi-protein complex under unchallenged conditions (Fig. 2c). Such a constitutive association might reflect a basal turnover of DDB2 in human cells. In any case, the observed interaction between p97

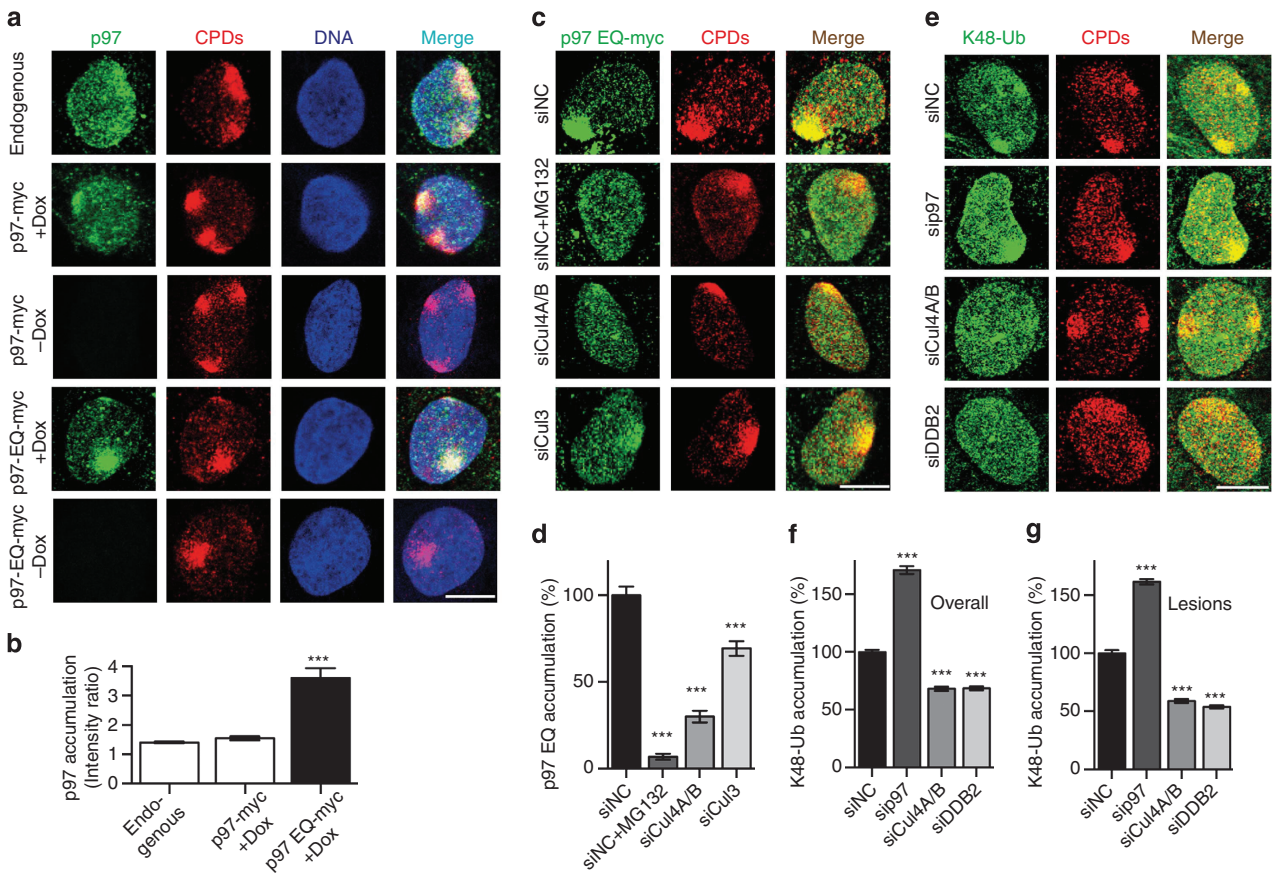

Figure 1 | Ubiquitin-dependent p97 recruitment to UV lesions. (**a**) Co-localization of p97 with CPDs in human cells. Mild expression of myc-tagged p97 (wild-type or p97 EQ) was induced with doxycycline (+Dox). Cells were probed 15 min after UV irradiation through micropore filters, whereby endogenous p97 was detected with anti-p97 antibodies and p97-myc with anti-myc antibodies. Scale bar, 10 μm. (**b**) UV-induced p97 re-localization shown as quantitative ratio of fluorescence at CPDs against surrounding nuclear areas (200 nuclei from two independent experiments); error bars, s.e.m., ***$P<0.001$ relative to wild-type (the unpaired two-tailed *t*-test was used for all *P*-value determinations). (**c**) Reduced recruitment of myc-p97 EQ to CPDs after treatment with MG132 or siRNA targeting the indicated cullins; siNC, non-coding control. (**d**) Quantification of p97 EQ at CPDs over three experiments (>300 nuclei), ***$P<0.001$ relative to siNC control (unpaired two-tailed *t*-test). (**e**) Increased K48-linked ubiquitin after treatment with siRNA targeting p97, CUL4A/B or DDB2. (**f**,**g**) Quantification (>300 nuclei from three experiments) of K48-linked ubiquitin in nuclei overall and at lesion sites, respectively.

and DDB2, the latter carrying K48-linked ubiquitin chains, was stimulated by UV radiation (Fig. 2c), supporting the notion that ubiquitinated DDB2 is a major p97 substrate upon UV exposure. This conclusion was supported by an inverse co-immunoprecipitation protocol, performed with the same HEK293 cells, whereby endogenous DDB2 was detected after pull-down of p97 EQ using Strep-Tactin beads that bind the Strep peptide of p97 (Fig. 2d).

The strong dependence on DDB2 (Fig. 2b) and the differential role of E3 ligases in recruiting p97 (Fig. 1d) suggested a critical involvement of this segregase in global-genome NER activity regulated by UV-DDB and Cul4A. In comparison, only a minor proportion of p97 localizing to UV damage is apparently contributing to transcription-coupled NER. This view was confirmed by depleting POLII whose stalling at UV lesions triggers Cul3-mediated degradation[30]. In fact, suppression of POLII levels reduced the UV-dependent recruitment of p97 to the same small extent as the Cul3 depletion (Fig. 2a,b).

Another scenario is that UV lesions block the DNA replication machinery[31,33,34,36] leading to degradation of the licensing factor CDT1 in a proliferating cell nuclear antigen (PCNA)- and CDT2-dependent manner[32,35]. However, depletion of none of these factors (PCNA, CDT1 or CDT2) reduced p97 recruitment to UV lesions (Fig. 2a,b), indicating that our assay, based on the induction of UV lesion spots, specifically monitors global-genome NER-related p97 functions.

The p97 segregase extracts DDB2 and XPC from chromatin. Next we tested how a depletion of p97 affects the homeostasis of DNA damage sensors in the NER pathway at the single-cell level. The siRNA-mediated downregulation of p97 resulted in increased recruitment (15 min after irradiation) and prolonged residence (180 min after irradiation) of DDB2 and XPC in UV-damaged areas (Fig. 3a–c), indicating that the p97 segregase extracts both sensors from chromatin. Retention of DDB2 in UV-irradiated chromatin was confirmed using cells expressing the p97 EQ mutant, which acts in a dominant-negative manner by generating inactive segregase complexes[37] (Supplementary Fig. 3a,b). That this segregase activity is dependent on prior substrate ubiquitination was additionally confirmed using a form of XPC that, as a result of its fusion with green fluorescent protein (GFP), is poorly ubiquitinated[17,39]. Unlike endogenous XPC (Fig. 3c), XPC-GFP was not removed from UV-damaged chromatin during the 180-min repair period (Supplementary Fig. 3c,d). The increased accumulation (after 15 min) and prolonged retention

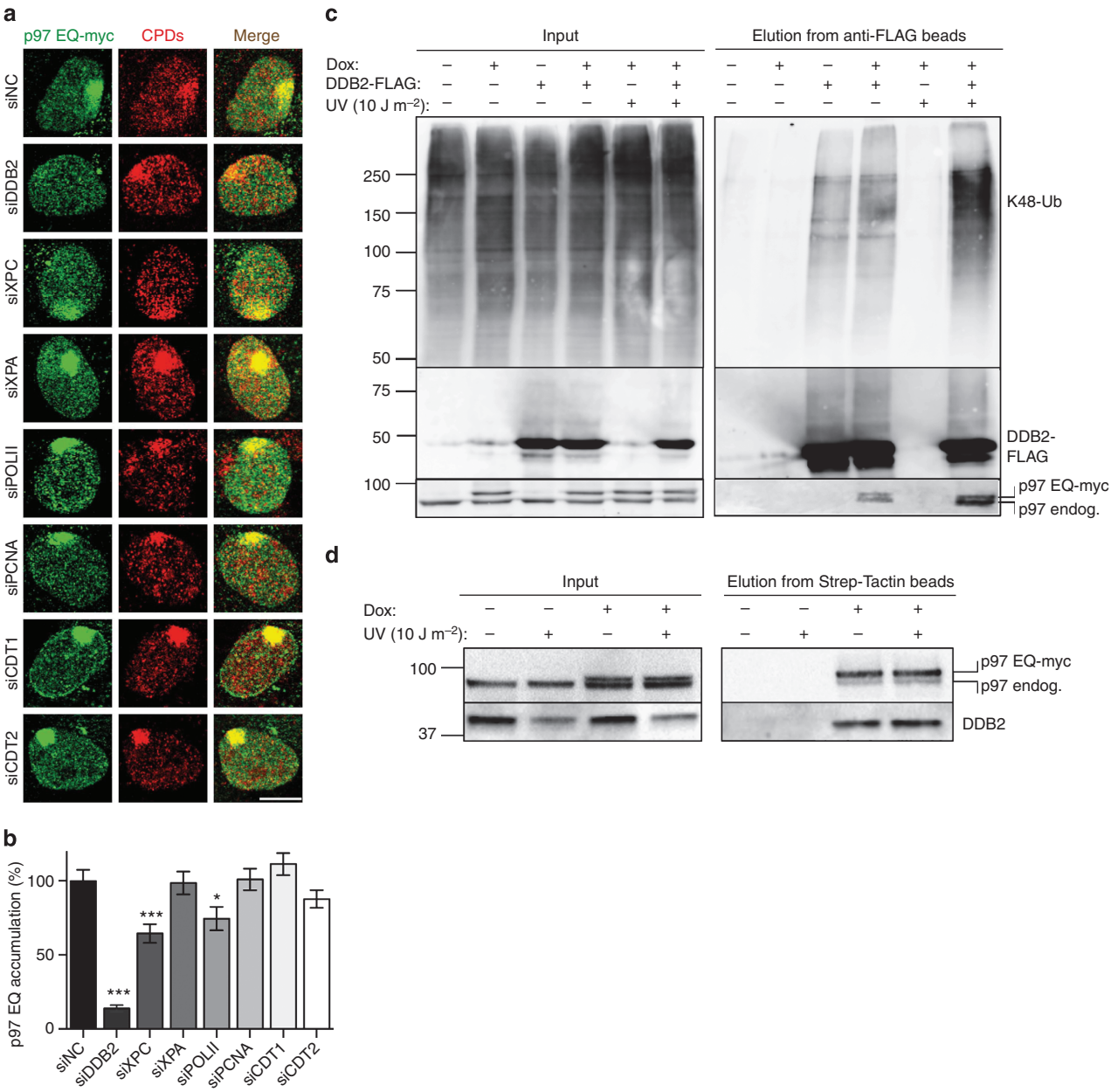

Figure 2 | Recruitment of p97 to UV lesions requires DDB2. (**a**) Co-localization of p97 EQ with CPDs in human cells treated with siRNA targeting the indicated proteins. Samples were probed 15 min after UV irradiation through micropore filters; scale bar, 10 μm. (**b**) Quantification of p97 EQ accumulation at CPDs as illustrated in Fig. 2a. Error bars, s.e.m. (>300 nuclei from three experiments); $*P<0.05$, $***P<0.001$ relative to siNC control (unpaired two-tailed *t*-test). (**c**) UV-stimulated interaction of p97 with a DDB2-containing complex. HEK293 cells were transfected to produce DDB2-FLAG and doxycycline-treated (Dox) for mild p97 EQ expression. DDB2-FLAG was pulled down from cell lysates using beads coated with anti-FLAG antibodies and eluates were analysed by immunoblotting against K48-ubiquitin, FLAG epitope and p97. Myc-tagged p97 migrates slower than the endogenous counterpart. (**d**) Interaction of DDB2 with the p97 complex. HEK293 cells were Dox for mild p97 EQ expression, and p97 EQ was pulled down from cell lysates using beads coated with Strep-Tactin. The resulting protein mixture was analysed by immunoblotting against p97 and DDB2.

(after 180 min) under conditions of inactivated p97 is unique to DDB2 and XPC, as these effects do not extend to downstream NER factors such as XPB (a subunit of the TFIIH complex; Supplementary Fig. 3e,f) or the ERCC1 subunit of the ERCC1-XPF endonuclease (Supplementary Fig. 3g,h).

The homeostasis of XPC and DDB2 was further investigated using biochemical methods. Upon UV irradiation, XPC protein is partially and reversibly ubiquitinated, with a maximal extent of modification around 1 h after exposure[16,19] (Fig. 3d, lane 3). Following p97 depletion, the proportion of ubiquitinated XPC was markedly increased and the ubiquitin modification remained for a longer time period of at least 6 h (Fig. 3d, lanes 6–10). Conversely, DDB2 rapidly transfers to chromatin after UV radiation and is then degraded by proteasomal activity[24] (Fig. 3d, lanes 1–5). This DDB2 clearance is inhibited by depletion of Cul4A (Supplementary Fig. 4a) or by depletion of p97 (Fig. 3d, lanes 6–10). In chromatin, the inhibition of DDB2 breakdown by p97 depletion occurs to a similar extent as in the presence of the proteasomal inhibitor MG132 (Fig. 3e, compare the chromatin-bound fractions of DDB2 in lanes 3 and 5). Accordingly, DDB2 persisted in the UV-damaged chromatin of p97-depleted cells (Fig. 3e, compare the chromatin-bound

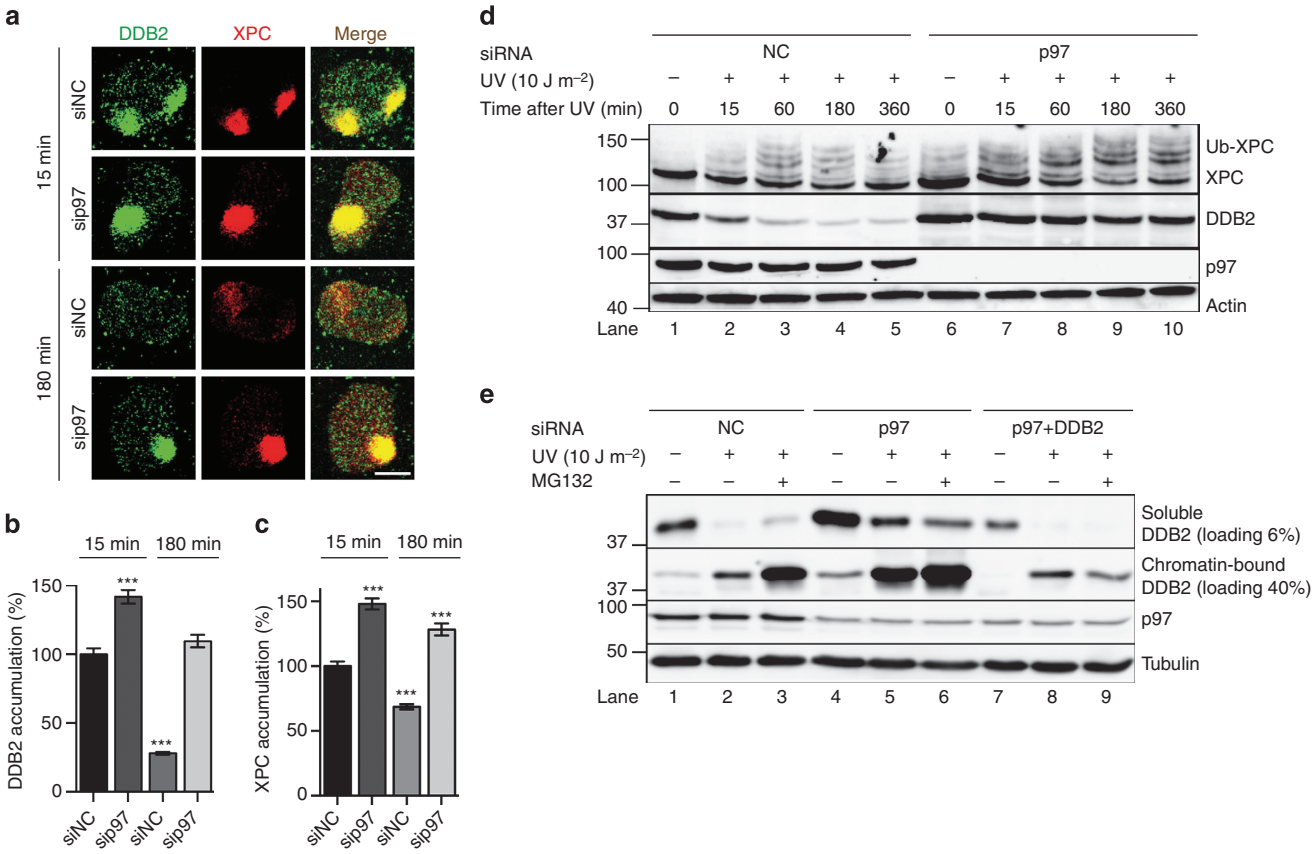

Figure 3 | The p97 segregase complex extracts DDB2 and XPC from chromatin. (**a**) Chromatin retention of DDB2 and XPC visualized 15 and 180 min after induction of UV damage spots in human cells treated with siRNA targeting p97 or non-coding (NC) control. Scale bar, 10 μm. (**b**,**c**) Quantification of DDB2 and XPC, respectively, in chromatin over three experiments (>300 nuclei). Error bars, s.e.m.; ***$P<0.001$ relative to siNC control (unpaired two-tailed *t*-test). (**d**) XPC hyperubiquitination and DDB2 degradation revealed by whole-cell immunoblot analysis after treatment with siRNA against p97 or NC control. (**e**) Levels of soluble (non-chromatin) and chromatin-bound DDB2 assessed in the respective cellular fractions.

fractions in lanes 2 and 5), whereas the combined depletion of p97 and DDB2 restored the lower level of DDB2 seen in control cells (Fig. 3e, compare the chromatin-bound fractions of DDB2 in lanes 2 and 8).

As the ubiquitination of DDB2 was not readily detected in cell lysates, to improve the visibility of this reaction, DDB2-FLAG was enriched by immunoprecipitation, under denaturing conditions, from transfected HEK293 cells using anti-FLAG beads. The resulting protein eluates were probed with antibodies against K48-linked ubiquitin, thus demonstrating that p97 depletion yields an increased level of ubiquitinated DDB2 relative to controls (Supplementary Fig. 4b,c). The stabilization of DDB2 against UV-induced degradation was confirmed using distinct siRNA sequences targeting p97 (Supplementary Fig. 4d) or by expressing the dominant-negative p97 EQ mutant (Supplementary Fig. 4e). In summary, these findings clearly show that p97 removes ubiquitinated DDB2 from UV lesions and thus facilitates its proteasomal breakdown.

Adaptors for the recruitment of p97 to UV lesions. Because the p97 segregase participates in multiple cellular processes, its substrate and site specificity is determined by adaptor proteins displaying both ubiquitin-binding and p97-interacting motifs[25,26]. Downregulation of the chromatin-related core adaptors Npl4 and Ufd1, by treatment with siRNA, completely abolished the recruitment of p97 to UV lesion spots (Fig. 4a,b). Instead, downregulation of UBXD1-mediating endolysosomal sorting did not interfere with this UV-dependent p97 recruitment. Similarly, downregulation of DVC1 (also known as Spartan or C1orf124) using the sequence siDVC_2, which resulted in efficient depletion (Fig. 4c), had no effect on the redistribution of p97 to UV lesion spots (Fig. 4a,b) despite the reported involvement of this adaptor in the DNA polymerase switch for translesion synthesis[31,33,34,36].

Consistent with the different importance of these adaptors for the p97 recruitment during global-genome NER activity, we found that depletion of Npl4 or Ufd1, but not downregulation of UBXD1 or DVC1, stabilized DDB2 protein in UV-irradiated cells to a similar extent as observed in the absence of p97 (Fig. 4c,d). Also, in full accordance with its role in triggering DDB2 degradation, we found that Ufd1 interacts with a DDB2-containing protein complex in HEK293 cells (Supplementary Fig. 4f). These studies also identified the UBXD7 adaptor as an additional factor in the homeostasis of NER factors, as its downregulation results in partial protection of DDB2 from UV-induced breakdown (Fig. 4d). Taken together, our findings demonstrate that p97, upon recruitment by specific adaptors, namely the Ufd1-Npl4 heterodimer and UBXD7, processes K48-ubiquitinated DDB2 in UV-damaged chromatin.

Excess of damage sensors reduces repair and genome stability. In view of this crucial role of the p97 complex in the homeostasis of DNA damage sensors, we tested its contribution to global-genome NER activity. By monitoring the excision of UV lesions

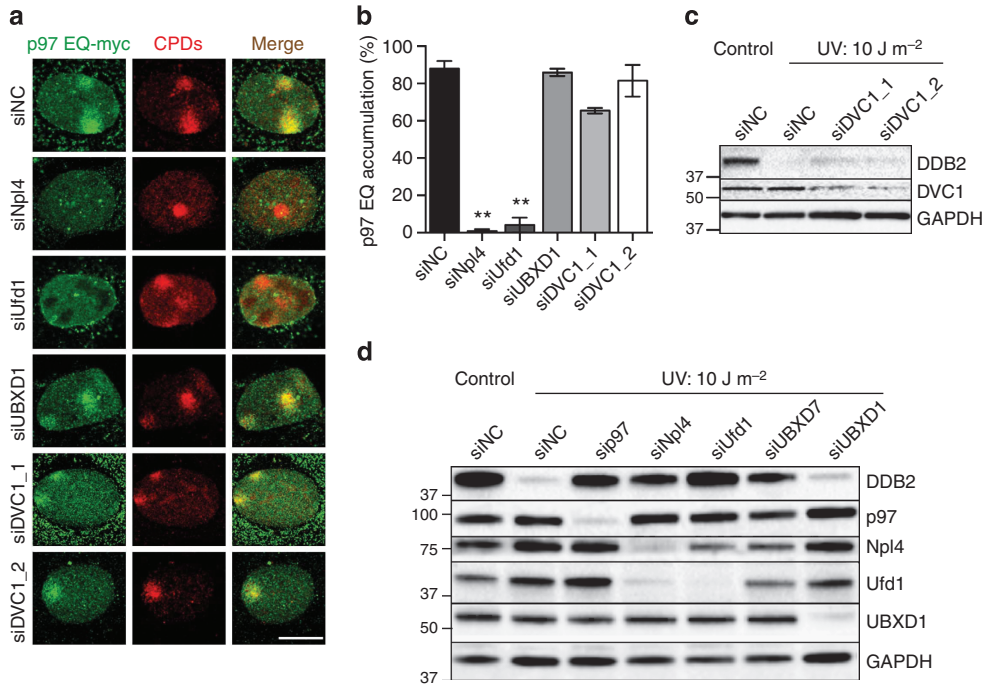

Figure 4 | Processing of DDB2 by the p97 segregase triggered by adaptors. (**a**) Co-localization of p97 EQ with CPDs in human cells treated with siRNA against the indicated adaptor proteins. The samples were probed 15 min after UV irradiation through micropore filters; scale bar, 10 μm. (**b**) Quantification of p97 EQ accumulation at CPDs as illustrated in Fig. 4a. Error bars, s.e.m. (>300 nuclei from three experiments); $^{**}P<0.01$, relative to siNC control (unpaired two-tailed *t*-test). (**c**) DDB2 degradation revealed by whole-cell immunoblot analysis after treatment with two different siRNA sequences against DVC1; siNC, non-coding control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase as loading standard. (**d**) Inhibition of UV-triggered DDB2 degradation in cells treated with siRNA targeting p97 or the indicated adaptors.

from chromosomal DNA, we observed that both the fast excision of 6-4PPs and the slow excision of CPDs[11,17] were attenuated by a p97 depletion in HeLa cells (Fig. 5a,b). Interestingly, the p97 downregulation caused inhibition of CPD excision in a similar manner as depletion of DDB2. That p97 functions as an accessory factor to facilitate NER activity explicitly in the physiologic chromatin context was supported by the finding that this segregase is not required for UV lesion excision from a transfected reporter plasmid (Supplementary Fig. 5a). This proficient repair of an ectopic and artificial substrate proofs that all NER factors are still present in p97-depleted cells, although they are unable to deploy their full activity on native chromosomes. Consistent with this compromised NER efficiency in the chromatin context, p97-depleted cells were hypersensitive to UV light (Supplementary Fig. 5b).

Given that the p97 segregase (i) processes K48-ubiquitinated DDB2 and XPC proteins; (ii) positively regulates NER activity in chromatin; and (iii) promotes cell survival after UV exposure, we finally tested whether this newly discovered regulation of DDB2 and XPC homeostasis contributes to genome stability by monitoring aberrations in metaphase chromosomes (Fig. 5c). We used HEK293 cells, unable to respond to UV damage with p53- and p21-dependent checkpoints[40], to exclude possible indirect and confounding effects from DNA damage-induced cell cycle arrest. An increased frequency of chromosomal aberrations occurred in p97-, DDB2- and XPC-depleted cells both under unchallenged conditions and, to a more pronounced degree, upon UV radiation (Fig. 5d; see Supplementary Fig. 5c for the corresponding protein levels).

Importantly, the genome instability following p97 depletion in UV-irradiated cells, but not in unchallenged cells, was reversed by concurrent downregulations, by siRNA, that reduce the level of DDB2 or XPC (Fig. 5d). These results suggested that a tight control of damage sensor levels in chromatin is essential for genome stability after UV radiation. As the siRNA-mediated depletion of DDB2 and XPC is not complete (see for example the remaining chromatin-bound fraction of DDB2 in Fig. 3e, lane 8), the reduced levels of these sensors are still adequate in a p97-deficient background to mediate a suitable NER response that prevents the formation of chromosomal aberrations upon infliction of UV damage.

The previous results indicate that an uncontrolled accumulation of DDB2 or XPC in chromatin is detrimental. If this hypothesis were correct, then excessive expression of such a damage sensor would be sufficient to cause genome instability in a background of normal p97 activity. This challenging concept was confirmed by demonstrating that overexpression of wild-type DDB2 but not overexpression of the DNA damage recognition-defective K244E mutant[41], both as FLAG-tagged constructs, inhibited the excision of 6-4PPs and CPDs (Supplementary Fig. 5d,e) and enhanced the frequency of chromosomal defects upon UV irradiation relative to control cells containing only the FLAG tag (Fig. 5e). Subsequently, double transfection experiments were carried out to induce the combined overexpression of both DDB2-FLAG and p97. The outcome of these double transfections (Fig. 5e) demonstrates that, by extracting the surplus of DDB2 from chromatin (see Supplementary Fig. 5f for DDB2-FLAG levels in and out of chromatin), a concomitant overexpression of the p97 segregase is able to reverse the chromosomal instability triggered by DDB2-FLAG present in excess. The enhancement of p97 amounts reached in these transfection-mediated overexpression experiments is illustrated in Supplementary Fig. 5g. To conclude, these findings show that increased levels of chromatin-bound DDB2 compromise genome stability only when they exceed the rate-limiting turnover capacity of the p97 segregase complex.

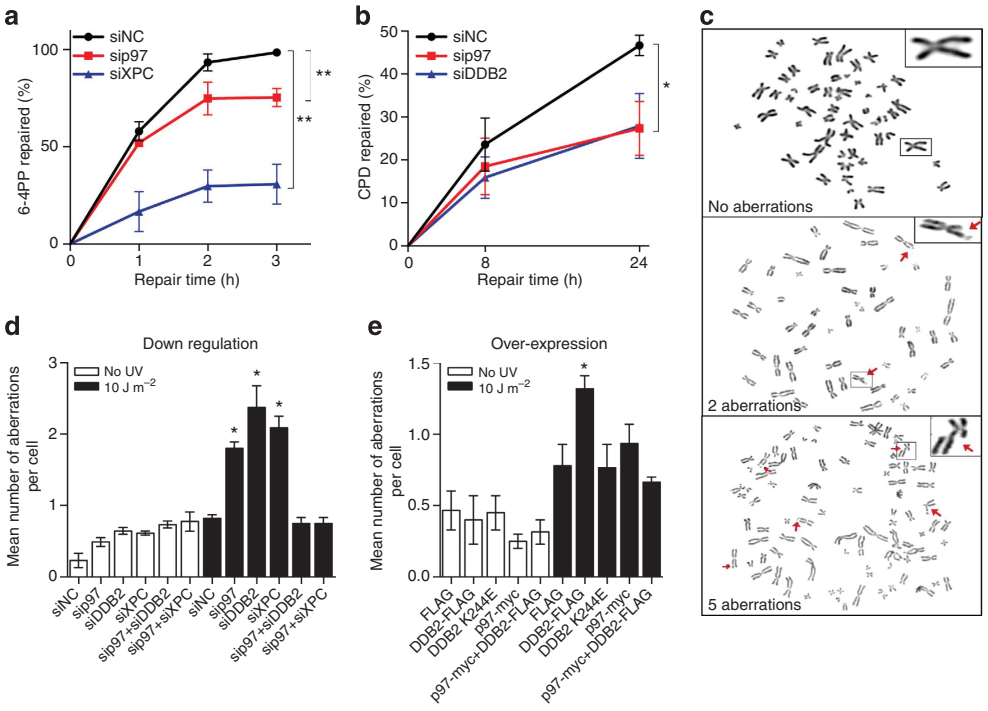

Figure 5 | An excess of damage sensors attenuates repair and causes genomic instability. (**a**) Excision of 6-4PPs in human cells treated with siRNA targeting p97 or XPC, used as comparator inflicting a severe repair defect; siNC, non-coding RNA control. Error bars, s.e.m. ($n=3$, each experiment with four replicates), $^{**}P<0.01$ (unpaired two-tailed t-test). (**b**) Excision of CPDs upon treatment with siRNA targeting p97 or DDB2 ($n=3$, each experiment with four replicates), $^{*}P<0.05$ (unpaired two-tailed t-test). In the case of CPDs, depletion of DDB2 is sufficient to compromise excision efficiency. (**c**) Metaphase spreads showing a progressively increasing genomic instability. Representative chromosomes are magnified in the inserts. (**d**) Chromosomal aberrations in cells treated with siRNA targeting the indicated proteins ($n=3$, 60 cells in a blinded analysis). $^{*}P<0.05$ relative to siNC and cells with double depletion (unpaired two-tailed t-test). (**e**) Chromosomal aberrations in cells overexpressing the indicated proteins ($n=3$, 60 cells in a blinded analysis). $^{*}P<0.05$ relative to cells expressing FLAG only and the double overexpressing cells (unpaired two-tailed t-test).

Discussion

This report identifies the p97 segregase machinery as a critical factor in the global-genome NER response that constitutes the main DNA repair system for protection from sunlight-induced skin mutagenesis and cancer. We show that the functional consequences of a downregulated p97 protein expression in human cells include UV hypersensitivity, a reduced rate of photoproduct excision and, consequently, an increased frequency of UV-induced chromosomal aberrations. The earliest DNA damage sensors in the global-genome NER pathway, DDB2, becomes a substrate of the p97 segregase when it is modified by Cul4A ligase-mediated ubiquitination in response to UV light. Depletion of XPC reduced the p97 accumulation at UV lesion sites, indicating that this simultaneously ubiquitinated partner is also a direct substrate of the p97 segregase. Thus, our findings imply that a key function of the ubiquitin modification is to prime these initial DNA damage sensors and NER initiators for subsequent extraction from chromatin. Along with UV-DDB and Cul4A ubiquitin ligase activity, recruitment of the p97 segregase to UV photoproducts depends on the ubiquitin-binding adaptors Npl4, Ufd1 and UBXD7, which are mediators of chromatin-associated p97 functions and, hence, confer substrate and site specificity to the p97 machine[25,26].

Even though DDB2 is needed for the efficient detection and excision of UV lesions, particularly of CPDs, ubiquitination leads to its degradation often within a few hours after exposure to UV light[24,42,43], but the actual trigger and scope of this apparently paradoxical breakdown of a lesion recognition subunit was unclear. Similarly, how the reversible ubiquitination of XPC protein influences the NER mechanism was poorly understood, although a widely accepted hypothesis is that this transient modification increases the binding affinity of XPC for DNA[16]. The newly identified role of p97 in the processing of ubiquitinated DDB2 and XPC provided a fresh strategy to investigate the impact of ubiquitin-mediated DDB2 degradation as well as the role of XPC ubiquitination. Our study is the first to show that, in the context of chromatin, a strict spatiotemporal control of the DNA lesion-associated homeostasis of DDB2 and XPC by the p97 segregase is critical for efficient NER activity (Supplementary Fig. 6). This conclusion is supported by the finding that overexpression of DDB2, resulting in prolonged binding of this damage sensor to lesion sites, inhibits the NER process rather than leading to a repair stimulation. Interestingly, the deleterious cellular end point (chromosomal aberrations) resulting from a deficient p97 function could be avoided by partial downregulation of DDB2 and XPC protein levels, confirming that an excess of these factors in the damaged chromatin of cells recovering from UV radiation is detrimental to genome stability. This is further demonstrated by the fact that overexpression of DDB2 is sufficient to boost up genome instability in UV-irradiated cells, and that this harmful outcome can be prevented by stimulating its degradation through concomitant p97 overexpression.

In summary, the paradigm of DDB2 homeostasis illustrates how both low and high levels of DNA damage sensors cause slow repair and genome instability (Supplementary Fig. 6). DDB2 stimulates the excision of UV lesions[11–14] but, if bound to damaged chromatin in excess because of a failure in its extraction and degradation, this same sensor acquires genotoxic properties culminating in chromosomal aberrations. Regulatory roles of DDB2 beyond its NER function have been identified in the

processes of apoptosis[44,45], cellular senescence[46], epithelial-to-mesenchymal transitions[47], breast tumour invasion[48] and alkylating drug resistance[49]. Therefore, the p97-directed control of DDB2 levels in damaged chromatin is of general importance for the understanding of genome dynamics, tumour development and cancer treatment[50].

Methods

Cell lines. Parental U2OS, HEK293 and HeLa cells, obtained from American Type Culture Collection, were grown in the dark using Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS; Gibco), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. In addition, 100 μg ml^{-1} hygromycin B and 100 μg ml^{-1} Zeocin were used in the case of stably transfected p97-myc U2OS cells, whereas 100 μg ml^{-1} hygromycin B and 15 μg ml^{-1} blasticidin S were used for stably transfected p97-myc-Strep HEK293 cells. U2OS cell lines, stably transfected with the inducible complementary DNA for wild-type p97 and p97 EQ, both displaying the myc epitope, were provided by Weihl and coworkers[51]. The corresponding HEK293 cells were designed as described in the Flip-In T-Rex 293 system protocol (R780-07; Invitrogen)[27]. Mild expression was achieved by induction with 1 μg ml^{-1} doxycycline for 24 h (wild-type p97) and 18 h (p97 EQ). All cell lines were tested negative for mycoplasma contamination.

siRNA transfections. The siRNA sequences were purchased from Qiagen, Microsynth or Invitrogen (Supplementary Table 1). Transfections were performed with Lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen). The siRNA concentrations were 16 nM for the silencing of p97, 20 nM for adaptors and 10 nM for all other targets (siRNA sequences are listed in Supplementary Methods). Cells were analysed 72 h after siRNA transfections.

Plasmids and cloning. The BamHI restriction was used to obtain the human *DDB2* complementary sequence from plasmid DDB2-GFP-C1 (a gift from Dr S. Linn, University of California, Berkeley, CA, USA) for insertion into the expression vector p3XFLAG-CMV-14 (Sigma). The K244E mutant[41] was generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene). The plasmid pcDNA5frt/to-p97wt for expression of wild-type p97 was described earlier[27]. A derivative resistant to siRNA against p97 was generated on the basis of the above-mentioned construct using QuickChange with the following primers: forward, 5′-AGCCGATTCAAAAGGTGATGATTTATCCACG GCTATCCTAAAGCAAAAGAACCG TCCCAATCGG-3′; reverse: 5′-CCGATT GGGACGGTTCTTTTGCTTTAGGATAGCCGT GGATAAATCATCACCTTTT GAATCGGCT-3′. Plasmid pEGFP-C1-Ufd1, used for ectopic expression of the Ufd1 adaptor, was a gift from Dr H. Meyer, University of Duisburg-Essen, Germany.

Plasmid transfections. DNA transfections were performed with FuGENE HD reagent according to the manufacturer's protocol (Roche). Cells were transfected at 20% confluency and analysed 72 h later, unless otherwise indicated in figure legends.

UV irradiation. Exposure of the cells to UV-C light was carried out with a germicidal lamp (wavelength 254 nm) after washing with phosphate-buffered saline (PBS). Local damage was generated by irradiation with 100 J m^{-2} through a 5-μm polycarbonate filter (Millipore)[52]. Cells were recovered for the indicated time periods under normal culture conditions before they were processed for further analyses. Protein synthesis was inhibited by addition of cycloheximide (100 μg ml^{-1}, Sigma) for 30 min before UV irradiation.

Immunofluorescence microscopy studies. Cells were grown on glass coverslips to 80% confluency. Pre-extraction buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% (v/v) Triton X-100) was added for 2.5 min at 4 °C. Thereafter, cells were fixed with 4% paraformaldehyde (w/v) for 15 min and permeabilized for 20 min with PBS containing 0.1% (v/v) Tween 20. Primary antibodies (listed in Supplementary Table 2) were diluted in PBS with 5% FCS and applied for 1 h at 37 °C after a 30-min blocking step with PBS containing 20% FCS. Secondary antibodies, diluted in PBS with 5% FCS, were added for 30 min at 37 °C after washing with PBS-Tween 20 and blocking for 20 min with PBS containing 20% FCS. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.2 μg ml^{-1}) for 10 min. Images of immunostained cells were taken with an SP5 confocal microscope (Leica) and analysed with the ImageJ software.

The accumulation of proteins at UV lesion sites was expressed as the ratio of fluorescence signal intensity at the damage site to the respective intensity in the remaining nuclear area after subtraction of the background intensity detected outside of the nucleus. Only in Fig. 1b, protein accumulation is presented directly as intensity ratios, whereas in all subsequent figures the results are expressed as the percentage of the ratio obtained with the indicated control treatment.

Immunoblotting. Cells were treated as indicated, washed with Puck's-EDTA and lysed in 100 μl of 1% Triton buffer (150 mM KCl, 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5% glycerol, 1 mM *N*-ethylmaleimide, 1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)). Protein concentrations were measured by Bradford analysis[53], Laemmli sample buffer[54] was added and boiled for 10 min at 95 °C. In each case, 50 μg of sample proteins were separated on 4–20% Criterion TGX stain-free precast gels (Bio-Rad) and transferred to polyvinyldene fluoride membranes using a Turbo transfer device (Bio-Rad). Antibodies used for immunoblotting are listed in Supplementary Table 2, and all full blots are shown in Supplementary Figs 7 and 8.

Chromatin digestion. Chromatin was dissected as described previously[17]. Briefly, cells were grown to confluency on 8.5 cm dishes, UV-irradiated with the indicated UV doses and lysed on ice with NP-40 buffer (25 mM Tris–HCl, pH 8.0, 0.3 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) NP-40, 0.25 mM phenylmethylsulfonyl fluoride and EDTA-free protease inhibitor cocktail (Roche)). Cell lysis was carried out for 30 min on a turning wheel. Free, non-chromatin-bound proteins were recovered by centrifugation (10 min, 15,000 *g*). Micrococcal nuclease (MNase; New England Biolabs) digestion was then accomplished for 20 min at 37 °C. For that purpose, the chromatin-containing pellet was suspended in CS buffer ((20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose and 0.1% (v/v) Triton X-100)) ref. 55 and supplemented with × 10 reaction buffer (500 mM Tris–HCl, pH 7.9, 50 mM $CaCl_2$), bovine serum albumin (1 mg ml^{-1}) and MNase (4 U μl^{-1}). Solubilized proteins were recovered by centrifugation (10 min, 15,000 *g*) after adding EDTA (5 mM) to stop the nuclease digestion.

Affinity purification of DDB2-FLAG complexes. Stable, inducible p97 EQ-Strep HEK293 cells were transfected with the vector for expression of DDB2-FLAG. The expression of p97 was induced by the addition of doxycycline as indicated in the figure legends. Cell fractions after MNase digestion were pooled (free non-chromatin-bound fraction containing soluble proteins and MNase-solubilized proteins), and the resulting mixture was incubated with anti-FLAG M2 affinity gel (Sigma) in the presence of immunoprecipitation buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1% (v/v) NP-40, 2 mM β-mercaptoethanol, 5% (v/v) glycerol, EDTA-free protease inhibitor cocktail (Roche) and 10 mM *N*-ethyl maleimide). The gel beads were washed with TNET buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100), TNE buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1 mM EDTA) and H_2O after an overnight incubation. The subsequent elution was performed with 0.3 μg μl^{-1} 3XFLAG peptide (Sigma) for 1 h at 4 °C on a shaker plate.

For the extraction of ubiquitinated DDB2-FLAG in denaturing conditions, cells were lysed (50 mM Tris–HCl, pH 6.8, 5 mM DTT and 1% (w/v) SDS) and sonicated before the solution was diluted four times with NP-40 buffer (25 mM Tris–HCl, pH 8.0, 0.3 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) NP-40, 0.25 mM phenylmethylsulfonyl fluoride and EDTA-free protease inhibitor cocktail (Roche)) and subjected to immunoaffinity purification with anti-FLAG M2 affinity gel as described above.

Affinity purification of p97 EQ-Strep complexes. Inducible p97 EQ-Strep from HEK293 cells was purified by Strep-Tactin sepharose beads (IBA Technologies) according to the manufacturer's protocol, in the presence of buffer containing 120 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.25% (v/v) NP-40, 2 mM mercaptoethanol, 5% (v/v) glycerol and protease/phosphatase inhibitor (Roche). After this pull-down, beads were washed, samples were boiled in loading buffer and the proteins were analysed by polyacrylamide gel electrophoresis and immunoblotting.

Quantification of UV lesions. To monitor the excision of UV lesions, antibodies against 6-4PPs and CPDs (Cosmo Bio) were used in a quantitative enzyme-linked immunosorbent assay[17] according to the manufacturer's instructions. Briefly, DNA purified from the whole genome (DNeasy Blood and tissue kit, Qiagen) was denatured by heating (99 °C, 10 min) followed by a 15-min incubation on ice. A volume of 50 μl per well of denatured DNA (4 μg ml^{-1} for 6-4PP detection, 200 ng ml^{-1} for CPD detection) was distributed into a 96-well microtiter plate (Greiner) coated with protamine sulphate (Sigma) and dried overnight at 37 °C. The DNA-coated plates were washed five times with PBST (0.05% (v/v) Tween 20 in PBS) and blocked with 2% FCS in PBS at 37 °C for 60 min. The antibodies against either 6-4PPs (64M-2) or CPDs (TDM-2) were used for 30 min (37 °C) at dilutions of 1:2,000 and 1:5,000, respectively. Primary antibodies bound to DNA molecules were recognized by biotin-labeled F(ab')$_2$ fragments of anti-mouse IgG (1:2,000; B-11027; Invitrogen) added for 30 min at 37 °C. After washing the plates, 100 μl of a peroxidase–streptavidin conjugate (1:10,000; Invitrogen) was distributed into each well. The reaction was started by adding 0.5 mg ml^{-1} *o*-phenylenediamine, 0.007% H_2O_2 and citrate-phosphate buffer (50 mM Na_2HPO_4, 24 mM citric acid, pH 5.0), stopped with 50 μl of 2 M H_2SO_4, and monitored by measuring the absorbance at 492 nm in a PLUS384 microplate spectrophotometer (Molecular Devices).

Chromosome analysis. HEK293 cells lacking p53- and p21-dependent checkpoint activation[40] were treated with siRNA for 72 h or, alternatively, transfected with overexpression plasmids. Cells were subsequently UV irradiated with 10 J m^{-2}, or left untreated, exposed to 100 nM nocodazole for 4 h, lysed with hypotonic solution (0.4% KCl) for 15 min and fixed in methanol:acetic acid (3:1). Cell suspensions were spread on a slide, air-dried and stained in Giemsa solution for 2 min. Metaphase spreads were detected using an Axio imaging 2 microscope (Zeiss, Jena, Germany) and captured using the Ikaros software (Metasystems, Altlusheim, Germany).

Host-cell reactivation assay. HeLa cells were grown to 10–20% confluency in a six-well dish and transfected with siRNA targeting the indicated proteins. Following 72 h, DNA transfections were carried out with 0.9 μg UV-irradiated pGL3 (254 nm wavelength, 1,000 J m^{-2}) and 0.1 μg non-irradiated pRL-TK (FUGENE HD, Roche). Cells were lysed after another 18 h incubation period according to the manufacturer's protocol (Dual Luciferase Assay, Promega). Ratios between *Photinus* and *Renilla* luciferase activity were determined in triplicates with a microtiter plate luminometer (Dynex).

Survival (colony-forming) assay. HeLa cells were transfected with siRNAs as previously described, UV-treated at increasing doses, seeded in different dilutions and incubated in cell culture medium for 7 days at 37 °C to allow for colony formation. Colonies were stained with 0.5% (w/v) crystal violet in 80% ethanol and counted.

Statistics. Mean values and s.e.m. are shown for each experiment. Differences between groups were calculated in GraphPad Prism 6 using unpaired, two-tailed, *t*-tests. *P* values expressed as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered to be significant. Between-group variances were similar and data were symmetrically distributed. In immunofluorescence studies, three independent experiments were performed and at least 100 random samples per experiment were scored in each group. To illustrate the sample size calculations for adequate statistical power, significance levels were set to 0.05, power levels to 0.9 and effect size to 0.5 according to the estimated mean values and s.d. The R statistical software version 3.0.1 was used to calculate sample size using two-sample *t*-test for power analysis (pwr package) yielding a sample size of 86.

Analogous power calculations were carried out for all other assays. In the enzyme-linked immunosorbent assay measurements, three independent experiments with four technical replicates were performed for each treatment. In chromosome analyses, 60 metaphase spreads were scored for chromosomal aberrations in three independent, blindly performed experiments. In colony-forming assays, three independent experiments with three technical replicates were performed for each treatment.

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Author contributions

M-R.P., D.L., P.R., N.K. and K.B. performed the experiments and interpreted the data; M-R.P. drafted the manuscript; K.R. and H.N. designed the study outline and wrote the final manuscript.

Additional information

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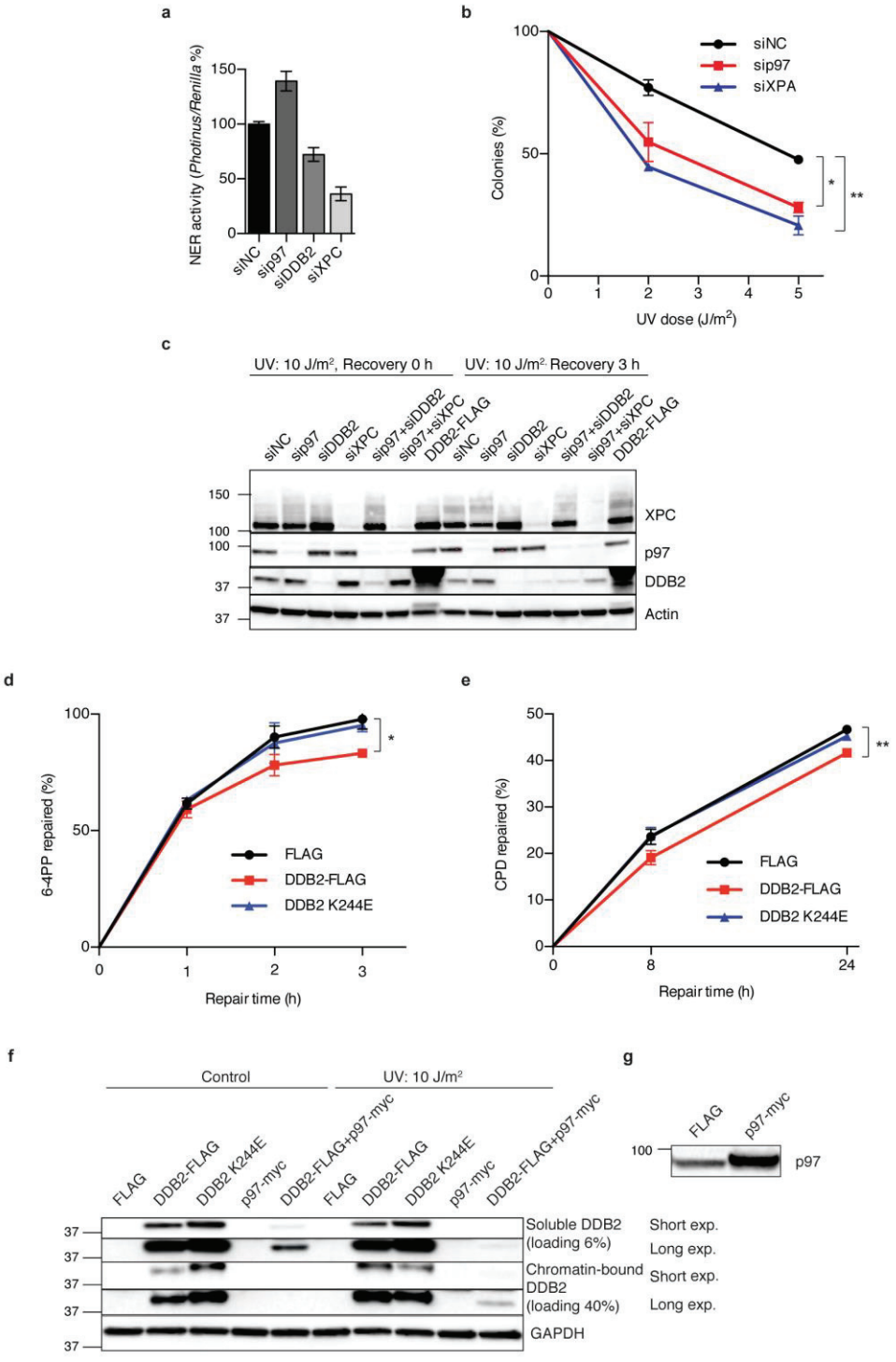
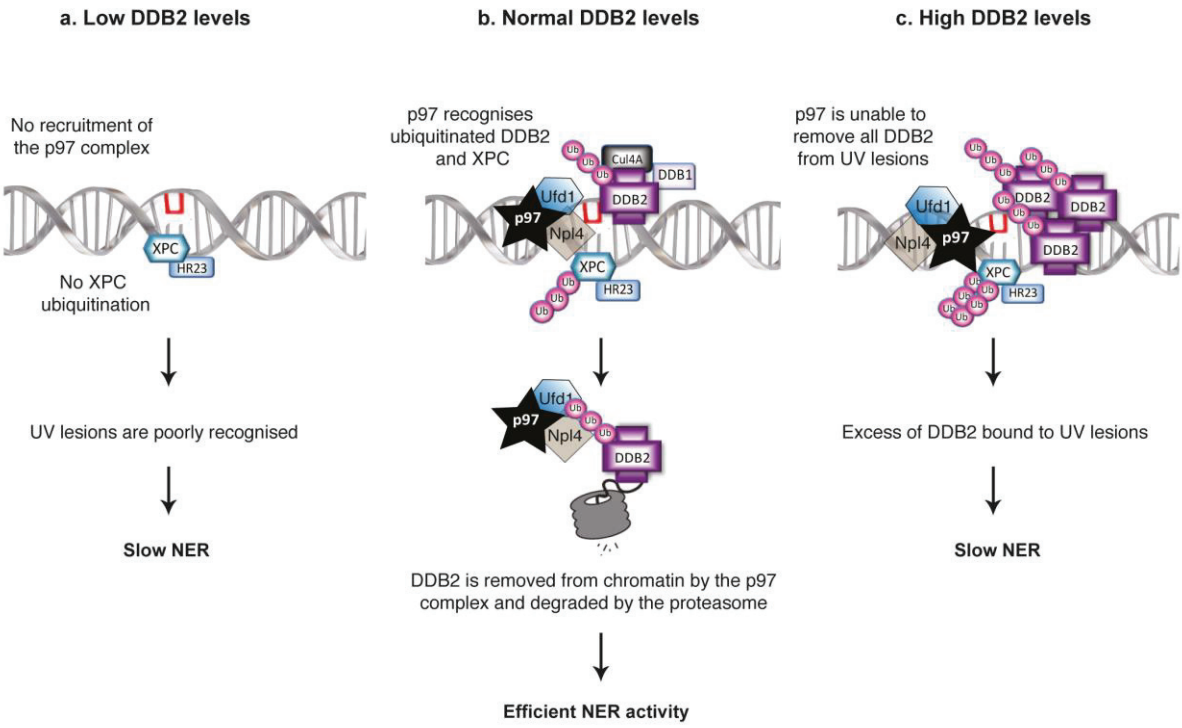


Supplementary Fig. 5. Down regulation of p97 causes hypersensitivity to UV light and chromosomal aberrations by disrupting DDB2 and XPC homeostasis.

(**a**) Host-cell reactivation of a luciferase reporter vector in HeLa cells treated with siRNA targeting the indicated proteins; siNC, non-coding control RNA. The assay was repeated

three times in triplicates; error bars, s.e.m. In this test based on a non-chromatinised DNA substrate, depletion of p97 resulted in slight stimulation of NER activity. (**b**) Colony forming assay demonstrating that p97-depleted HeLa cells (by treatment with siRNA targeting p97) are hypersensitive to killing by UV radiation (n=3, each measurement in triplicate). The error bars indicate s.e.m. (**P<0.01 and *P<0.05 using the unpaired two-tailed t-test). (**c**) Whole-cell level of the indicated proteins after siRNA treatment (72 h) of HEK cells. In part, the cells were transfected with a vector for expression of DDB2-FLAG as indicated. (**d, e**) Excess of DDB2 but not of the K244E mutant, resulting from transfection of HeLa cells with the respective expression vectors, slows down repair of 6-4PP and CPDs (n=4, each measurement in duplicate). The error bars indicate s.e.m. (**P<0.01 and *P<0.05 using the unpaired two-tailed t-test). (**f**) Levels of DDB2 protein bound to chromatin and in the non-chromatin ("soluble") fraction of HEK cells after over-expression of p97-myc, as indicated. The samples for immunoblotting were collected before UV irradiation and 3 h after irradiation. (**g**) Over-expression of p97-myc in HEK cells. The control lane ("FLAG") shows the normal level of endogenous p97 in cells transfected with a vector coding for the FLAG epitope. The lane indicated with "p97-myc" illustrates the increased p97 level induced by transfection with the respective expression vector. The blot was probed with anti-p97 antibodies.



Supplementary Fig. 6. Model illustrating how DDB2 levels, regulated by the p97 segregase complex, impact on NER efficiency. (**a**) In the absence of DDB2, UV lesions are poorly recognized and their excision is slow. Also, XPC is not ubiquitinated and the p97 complex is not recruited to UV lesion sites. **(b)** Efficient global-genome NER activity requires both normal levels of DDB2 and the p97-dependent removal of this damage sensor from its DNA substrate in chromatin. **(c)** Conversely, excessive levels of DDB2 on the DNA substrate in chromatin lead to impaired NER activity and, as a consequence, to genome instability.

3.3 The CHD1 remodeler displaces XPC-nucleosome intermediates during DNA nucleotide excision repair

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In the following manuscript we could show that active chromatin remodelling by the ATPase CHD1 supports the NER activity. I designed and analysed the experiments. The experiments have been performed by *Z. Garajovà, C. Balbo Pogliano and P. Rüthemann*. I prepared all figures and drafted the manuscript together with H. Nägeli.

3.3.1 Abstract

Ultraviolet (UV) light induces mutagenic cyclobutane pyrimidine dimers (CPDs) embedded in chromatin, where DNA is wrapped around histone octamers forming nucleosomes. How global-genome nucleotide excision repair (GG-NER) processes CPDs despite this chromatin arrangement is poorly understood. By immunoprecipitation of nucleoprotein complexes from fragmented chromatin, we found that the remodeler Chromodomain helicase DNA-binding 1 (CHD1) co-localizes transiently with GG-NER factors after UV irradiation. Follow-up chromatin fractionations confirmed that CHD1 is recruited to UV lesions on nucleosomes, consistent with a role in displacing these nucleosomes to allow for excision repair. Protein depletion studies revealed that the UV-dependent relocation of CHD1 to nucleosomes requires the xeroderma pigmentosum group C (XPC) sensor for lesion demarcation on nucleosomes. Thus, against the generally contended notion that the XPC complex is unable to access histone octamer-bound substrates, nucleosomes actually provide a structural scaffold that facilitates the recognition of CPDs by XPC protein. Chromatin remodeling is then required to convey the resulting XPC-nucleosome intermediates to the GG-NER pathway, as demonstrated by the finding that CHD1 triggers the displacement of XPC protein from lesion sites and promotes the recruitment of downstream factors, thereby stimulating CPD excision and protecting from UV-induced cytotoxicity.

3.3.2 Introduction

Genomic DNA is highly susceptible to damage caused by a plethora of endogenous or environmental genotoxic agents. In particular, bulky base lesions induced by UV light and the consequent accumulation of mutations are the major cause of skin cancer (Mouret *et al*, 2011; Marteijn *et al*, 2014; DiGiovanna & Kraemer, 2012). UV irradiation of DNA gives rise to cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) in a ratio of ~3:1 (Kobayashi *et al*, 2001). The quantitatively predominant CPDs are distributed evenly in chromatin and, hence, arise abundantly in nucleosome cores where the DNA is wrapped around histone octamers (Smerdon & Conconi, 1999; Zavala *et al*, 2014; Han *et al*, 2016). Nucleotide excision repair (NER) is the versatile multi-step process that removes these UV lesions as well as other bulky base adducts elicited by chemical carcinogens or oxygen radicals. Depending on their genomic location, bulky lesions are sensed by two alternative mechanisms. In the template strand of transcribed genes, detection of DNA damage occurs when the elongating RNA polymerase II complex encounters obstructing lesions (Hanawalt & Spivak, 2008; Vermeulen & Fousteri, 2013). Conversely, global-genome NER (GG-NER) detects bulky DNA adducts anywhere in the genome independently of transcription (Sancar, 1996; Hoeijmakers, 2009; Schärer, 2013). Genetic defects in the latter pathway result in the cancer-prone syndrome xeroderma pigmentosum (XP) with patients being classified into complementation groups reflecting mutations in distinct GG-NER genes (Friedberg *et al*, 2006).

The GG-NER reaction relies on a trimeric complex consisting of XPC, RAD23B (a human homolog of yeast RAD23) and centrin 2 to initially sense the presence of bulky lesions in the DNA double helix (Sugasawa *et al*, 1998; Araki *et al*, 2001; Volker *et al*, 2001). The DNA-binding function of this initiator complex resides entirely with the XPC subunit that is additionally supported in its lesion recognition activity by interactions with UV-damaged DNA-binding (UV-DDB) protein, also known as DDB1-DDB2 heterodimer (Hwang *et al*, 1999; Tang *et al*, 2000; Wakasugi *et al*, 2001; Rapic-Otrin, 2002; Fitch *et al*, 2003). The XPC subunit mediates recruitment of the transcription factor IIH (TFIIH) complex, which contains the XPD helicase that scans DNA for damage verification and concomitantly unwinds the double helix by 20-25 nucleotides around the lesion (Evans et al. 1997; Riedl *et al*, 2003). The transiently unwound state is then stabilized by XPA in conjunction with replication protein A (RPA) (Li *et al*, 2015), until the endonucleases XPG and XPF/ERCC1 (a heterodimer of XPF and excision repair cross-complementing 1) incise the damaged strand on each side of the unwound duplex and thus remove damaged bases as part of an excised oligonucleotide (Araújo *et al*, 2000; Reardon & Sancar, 2003; Staresincic *et al*, 2009). After oligonucleotide excision, the remaining single-stranded gap is filled by DNA synthesis and closed by DNA ligation (Ogi *et*

al, 2010; Moser *et al*, 2007). How this multi-step process takes place despite compaction of the DNA substrate in chromatin, is under intense scrutiny, but not yet understood.

Members of distinct families of ATP-dependent remodelers have been implicated in chromatin relaxation to allow for GG-NER activity. A pioneer study in yeast indicated that switch/sucrose non-fermenting (SWI/SNF) stimulates GG-NER activity in transcriptionally silent loci (Gong *et al*, 2006). In higher eukaryotes, the DDB1-DDB2 heterodimer has been shown to recruit at least three chromatin remodelers, i.e., Brahma-related gene 1 (BRG1, a catalytic subunit of SWI/SNF) (Zhang *et al*, 2009; Zhao *et al*, 2009), Amplified in liver cancer 1 (ALC1) (Pines *et al*, 2012) and Inositol requiring 80 (INO80) (Jiang *et al*, 2010). In a further study, however, INO80 was not required for chromatin remodeling before initiating GG-NER activity, but for the restoration of nucleosome repeats after DNA repair (Sarkar *et al*, 2010). In addition, the mammalian SWI/SNF subunit SNF5 (for sucrose non-fermenting 5) interacts with XPC protein and its deletion causes UV hypersensitivity (Klochendler-Yeivin *et al*, 2006; Ray *et al*, 2009), although these results were challenged by another study where no effect of SNF5 on UV sensitivity was detected (McKenna *et al*, 2008). It is clear from the above reports that chromatin remodelers facilitate the GG-NER process although the underlying mechanisms remain controversial (Aydin *et al*, 2014b). The basic conundrum remains whether chromatin relaxation precedes DNA lesion detection or vice versa (Rubbi & Milner, 2003). It is also not known if remodeling machines are recruited to unfold nucleosomes for the access of GG-NER factors to DNA, or rather drive the assembly of repair complexes onto DNA, or their disassembly from DNA, after chromatin relaxation by other mechanisms. In addition, previous reports on BRG1 and SNF5 show that chromatin remodelers may assist the GG-NER reaction by inducing cell cycle checkpoint signaling (Ray *et al*, 2009; Zhang *et al*, 2013) or that their depletion may lead to apoptosis, which attenuates GG-NER activity (Gong *et al*, 2008).

Chromodomain helicase DNA-binding protein 1 (CHD1) has been shown to promote chromatin plasticity that is crucial for the pluripotency of embryonic stem cells, for transcriptional reprogramming and homologous recombination (Park *et al*, 2014; Nodelman *et al*, 2016; Gaspar-Maia *et al*, 2009; Piatti *et al*, 2015; Kari *et al*, 2016). The genome-wide range of these known functions of CHD1 prompted us to test whether this same remodeler is also involved in chromatin dynamics required for GG-NER activity. We found that, in UV-damaged chromatin, CHD1 stimulates the turnover of XPC protein at DNA lesion sites in exchange with TFIIH and further downstream factors like the XPA subunit. To facilitate this handover, XPC protein recruits CHD1 directly to nucleosome cores independently of the DDB2 partner, indicating that XPC protein on its own is able to form a lesion recognition intermediate with damaged DNA wrapped around histone octamers. The main implication of this unexpected mechanism is that, rather than always representing a barrier impeding the

accessibility to damaged DNA, nucleosomes play an active scaffolding role in priming specific lesions for the GG-NER reaction.

3.3.3 Results

CHD1 co-localizes in chromatin with GG-NER factors

To test whether CHD1 associates with UV-damaged chromatin and GG-NER complexes during DNA repair, we transiently transfected HEK293 cells with a construct that drives overexpression of the DDB2 subunit of UV-DDB fused to the FLAG peptide. The purpose of this approach was to exploit the tight binding of DDB2 protein to UV-damaged DNA (Rapic-Otrin, 2002; Yeh *et al*, 2012) and, concomitantly, its transient interactions with the core GG-NER factors XPC and XPA (Sugasawa *et al*, 2005; Wakasugi *et al*, 2009). These two subunits, in turn, associate with each other and also with the TFIIH complex comprising the XPD helicase (Nocentini *et al*, 1997; Yokoi *et al*, 2000; Uchida *et al*, 2002; Bunick *et al*, 2006). The DDB2-FLAG fusion protein was, therefore, used as a molecular bait to isolate short chromatin fragments containing both UV lesions and GG-NER factors and to test whether CHD1 co-localizes in these same nucleoprotein complexes.

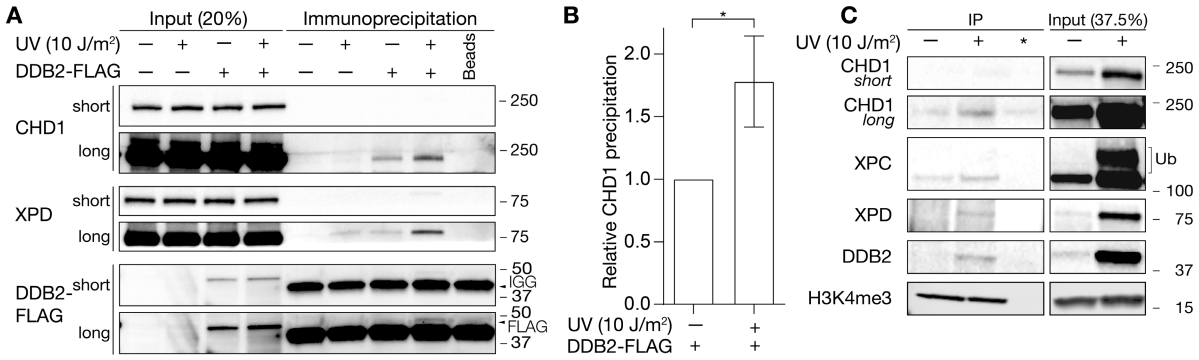

Figure 1 ***CHD1 co-localizes in chromatin with GG-NER proteins.*** *(A) HEK293 cells were transfected with a vector for expression of FLAG-tagged DDB2 and treated with UV-C light. Chromatin was salt-extracted and, after fragmentation by micrococcal nuclease (MNase), dissolved by sonication. The FLAG tag was then used to precipitate DDB2 with anti-FLAG antibodies. Input fractions and immunoprecipitated complexes were analyzed by blotting with antibodies against CHD1, DDB2 and XPD (short and long exposures are shown). IGG, immunoglobulin G heavy chains interfering with the detection of DDB2-FLAG. CHD1 is a protein of 197 kDa that, in polyacrylamide gels, migrates to a position just below the 250-kDa marker. (B) Quantified CHD1 levels co-localizing in chromatin with NER complexes, normalized to the amount of CHD1 in the respective inputs (n = 3 independent experiments). (C) Immunoprecipitation of histone H3 (using anti-H3K4me3 antibodies) from the fragmented chromatin of U2OS cells. Immunoprecipitated (IP) complexes and input fractions were analyzed by immunoblotting with antibodies against CHD1, XPC, XPD, DDB2 and H3K4me3. The asterisk denotes a control sample where uncoated protein G-sepharose beads were incubated with the fragmented chromatin of UV-irradiated cells. Ub, ubiquitinated XPC.*

*Data information: In B, data are presented as mean ± SEM. *P ≤ 0.05 (one-sample t-test with a hypothetical value of 1.0).*

A critical step for the detection of a UV-dependent recruitment of CHD1 to chromatin was its pre-extraction with salt (0.3 M NaCl) to remove free proteins that are not or only loosely associated with chromatin. The pre-extracted chromatin was then dissected by digestion with saturating amounts of micrococcal nuclease (MNase), which cleaves DNA preferentially in linker segments spacing the nucleosome cores (Supplementary Figure 1). Finally, the fragmented chromatin as a whole was solubilized by sonication before carrying out immunoprecipitation studies using anti-FLAG antibodies. Analysis of the immunoprecipitated chromatin complexes confirmed that the XPD subunit (as an example of core NER factor) localizes to chromatin in a UV-dependent manner (Figure 1A).

A substantial amount of XPD was co-immunoprecipitated from the chromatin fragments of cells that were transfected with the DDB2-FLAG construct and, subsequently, UV-irradiated. Instead, essentially no XPD was immunoprecipitated from the chromatin of cells without DDB2-FLAG or in the absence of prior UV irradiation. Importantly, CHD1 was found within the same chromatin complexes containing DDB2-FLAG and this recruitment of CHD1 to chromatin increased markedly with the induction of UV damage (Figure 1A). Quantification of CHD1 levels in immunoprecipitated complexes, using CHD1 in the respective input fraction as the reference, showed its preferential co-localization with GG-NER factors in response to UV irradiation (Figure 1B).

To confirm this co-localization in chromatin, we exploited the affinity of the chromodomains of CHD1 for histones (Flanagan *et al*, 2005). For that purpose, chromatin from U2OS cells was pre-extracted, fragmented and sonicated as described above, and the resulting chromatin fragments were subjected to immunoprecipitation using antibodies against histone H3. Inspection of the immunoprecipitated complexes demonstrated that the sequestration of CHD1 in chromatin is increased upon UV irradiation (Figure 1C). Also, this approach confirmed that the GG-NER proteins XPC, XPD and DDB2 co-immunoprecipitate with CHD1, consistent with a co-localization of CHD1 with GG-NER factors in the chromatin of UV-irradiated cells (Figure 1C).

CHD1 is recruited to UV-damaged nucleosome cores

Instead of direct solubilization by sonication, the chromatin fragments obtained from U2OS cells were fractionated to delineate the positions at which CHD1 co-localizes with GG-NER factors. As above, free proteins that are not or only loosely associated with chromatin were removed by salt (0.3 M NaCl) extraction and the remaining chromatin was dissected by incubation with MNase. Notably, at saturating levels of this nuclease, the genome is totally converted to short DNA segments of 147-base pairs (Figure 2A). The size of these residual fragments corresponds to the DNA length of nucleosome cores protected from MNase

digestion by interactions with histone octamers. Thus, saturating MNase digestions reduce the entire chromatin to nucleosome cores by eliminating all internucleosomal linker DNA segments.

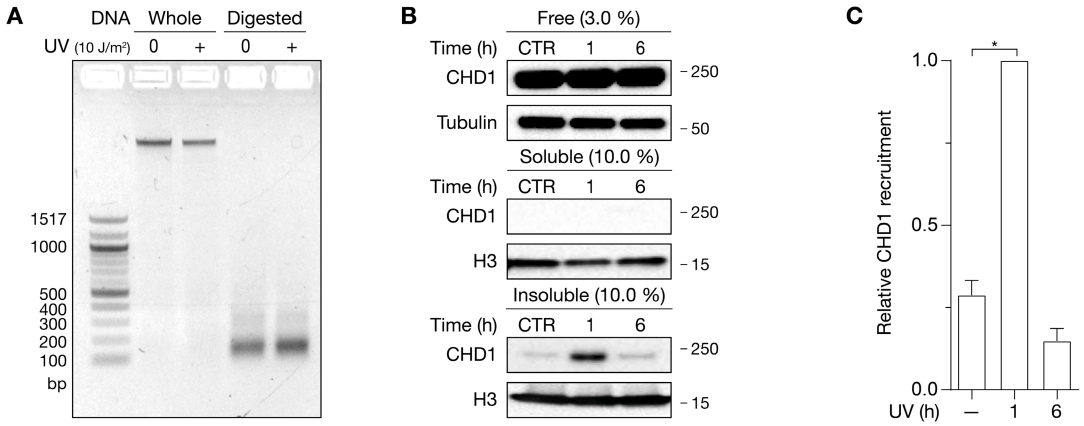

Figure 2 ***CHD1 is recruited to nucleosome cores upon UV irradiation.*** *(A) The chromatin of U2OS cells, untreated or UV-irradiated, was fragmented by MNase digestion (4 U/µl). A subsequent agarose gel analysis demonstrates the complete breakdown of internucleosomal linker DNA segments resulting in residual chromatin containing exclusively nucleosome core fragments of 147 base pairs. (B) In response to UV irradiation (10 J/m²), CHD1 is transiently recruited to nucleosome cores. The chromatin of U2OS cells was salt-extracted and MNase-digested (4 U/µl) to generate, as shown in the three panels from top down, a fraction of free (or loosely chromatin-bound) proteins, a fraction of solubilized chromatin proteins and a fraction of condensed and, hence, insoluble nucleosome cores. CTR, unirradiated control. Histone H3 and tubulin serve as the loading standards. (C) Quantification of CHD1 recruitment to nucleosome cores, normalized to the total level of CHD1, following 1 and 6 h after UV irradiation (10 J/m²) (n = 4 independent experiments).*
*Data information: In C, data are presented as mean ± SEM. *P ≤ 0.05 (one-sample t-test with a hypothetical value of 1.0).*

Analysis of the different fractions showed that most CHD1 is found in an unbound state as free protein that is extracted in the presence of 0.3 M NaCl (Figure 2B, top panel). The subsequent MNase digestion of pre-extracted chromatin generates a soluble supernatant of proteins released from chromatin that also includes some dissociated nucleosome cores containing *inter alia* histones H3 but essentially no CHD1 (Figure 2B, middle panel). However, the vast majority of nucleosome cores remains in a condensed and, hence, insoluble form even after digestion with saturating MNase concentrations (Supplementary Figure 1). We observed that a proportion of CHD1 protein is immobilized in this nucleosome core-enriched (insoluble) fraction upon UV irradiation (Figure 2B, bottom panel). This UV-dependent recruitment of CHD1 to nucleosome cores is observed around 1 h after UV irradiation but, subsequently, CHD1 disappears from this chromatin localization within 6 h after UV irradiation.

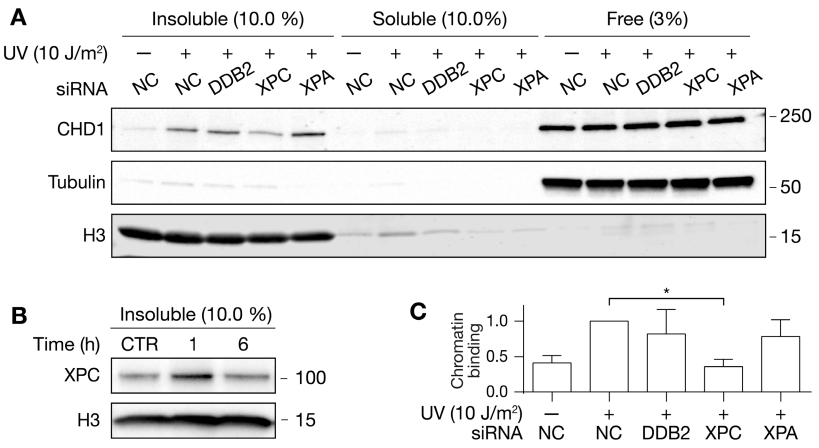

Figure 3 ***CHD1 is recruited to nucleosome cores by the XPC initiator.*** *(A) XPC-dependent recruitment of CHD1 to nucleosome cores. U2OS cells were siRNA-transfected as indicated two days before irradiation with UV (10 J/m²), or before mock treatment, and incubated for another 3 h. Chromatin was salt-extracted and MNase-digested to generate, from left to right, an insoluble fraction of condensed nucleosome cores, a fraction of solubilized chromatin proteins and a fraction of free (or loosely chromatin-bound) proteins. NC, non-coding control RNA. Histone H3 and tubulin serve as the loading standards. (B) Transient association of XPC protein with nucleosome cores in response to UV irradiation. CTR, mock-treated cells. (C) Quantification of CHD1 recruitment to nucleosome cores normalized to the level of CHD1 in control reactions with non-coding RNA (n = 5 independent experiments).*
*Data information: In C, data are presented as mean ± SEM. *P ≤ 0.05 (one-sample t-test with a hypothetical value of 1.0).*

XPC-dependent recruitment of CHD1 to nucleosomes

Next, different GG-NER factors were depleted in U2OS cells by transfection with small interfering RNA (siRNA) to test the mechanism by which CHD1 is recruited to nucleosome cores. The efficiency of each siRNA-mediated down regulation is demonstrated by immunoblotting (Supplementary Figure 2). These depletion experiments revealed that the UV-dependent recruitment of CHD1 to nucleosome cores was reduced by depletion of XPC protein (Figure 3A). Consistent with this dependence on XPC, we confirmed as previously reported (Fei *et al*, 2011) that the XPC subunit itself is recruited to this same nucleosome core fraction in a transient manner, i.e., with a time course comparable to that observed with CHD1 (Figure 3B). Depletion of DDB2, an accessory subunit that is active in the GG-NER pathway upstream of XPC, does not affect the UV-dependent relocation of CHD1 to nucleosome cores. Similarly, depletion of XPA, a core subunit acting in the GG-NER pathway downstream of XPC, did not influence this UV-dependent relocation of CHD1 to nucleosome cores (Figure 3C).

CHD1 stimulates XPC displacement and recruitment of downstream GG-NER factors

U2OS cells were depleted of CHD1 using siRNA to test the impact of this chromatin remodeler on the GG-NER reaction. This down regulation reduced the level of CHD1 protein by ~80% within two days after transfection with siRNA (Figure 4A and Supplementary Figure 2).

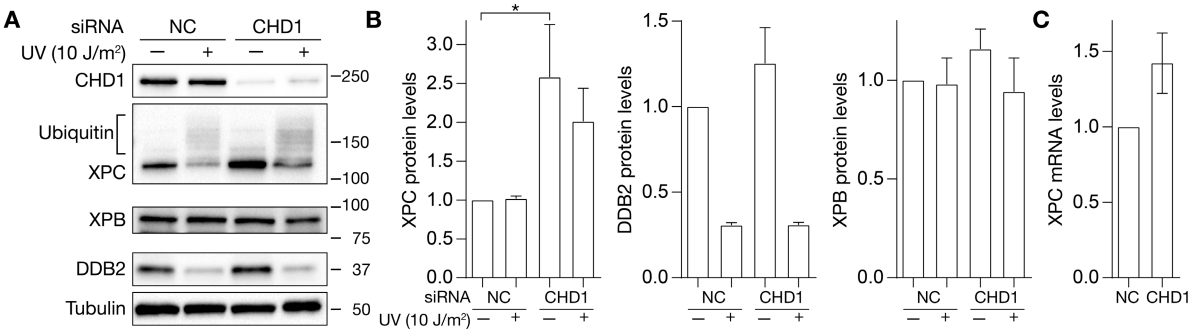

Figure 4 ***Depletion of CHD1 by siRNA treatment.*** *(A) Effect of CHD1 on GG-NER protein levels. U2OS cells were transfected with non-coding control RNA (NC) or siRNA against the CHD1 transcript and tested after 2 days. Cells were harvested for analysis 1 h after UV exposure or mock treatment. Immunoblots of whole cell lysates were carried out with the indicated antibodies. The higher molecular weight forms of XPC protein reflect its known ubiquitination by the CRL4^{DDB2} ligase (Sugasawa et al, 2005). Similarly, the ubiquitin-dependent proteasomal degradation of DDB2 is a well-described reaction to UV irradiation. Tubulin served as the loading control. (B) Quantification of XPC, DDB2 and XPB protein levels determined by immunoblotting 2 days after transfection with siRNA (n = 3-6 independent experiments). The UV-irradiated samples were analyzed 1 h after treatment. (C) Increased level of mRNA coding for XPC resulting from CHD1 depletion 2 days after transfection with siRNA (n = 3 independent experiments).*
*Data information: In B, C, data are presented as mean ± SEM. *P ≤ 0.05, **P < 0.01 (one-sample t-test with a hypothetical value of 1.0).*

However, this substantial reduction of CHD1 protein did not affect the cell division cycle of unchallenged cells (Supplementary Figure 3) and did not trigger any apoptotic responses leading to activation of caspase 3 (Supplementary Figure 4). In view of its established role in transcription (Simic *et al*, 2003; Smolle *et al*, 2012; Park *et al*, 2014), it could have been expected that the down regulation of CHD1 may interfere with DNA damage processing by diminishing the expression of repair proteins. Figure 4A shows that the CHD1 depletion does not reduce the cellular level of GG-NER factors like XPB, XPC or DDB2 measured two days after transfection with siRNA. On the contrary, we consistently observed that cells respond to CHD1 depletion with a constitutively increased level of XPC protein (see the quantification of protein levels in Figure 4B). The high molecular weight forms of XPC in the immunoblot of Figure 4A (representing ubiquitinated XPC protein) also indicate that the CHD1 down regulation does not detectably interfere with the UV-dependent ubiquitination reaction. The CHD1 depletion similarly does not impair the well-described degradation of DDB2 in response to UV irradiation (Figures 4A and 4B). The observed up regulation of XPC under conditions of a CHD1 deficiency already takes place at mRNA levels as demonstrated by quantitative reverse-transcription PCR measurements (Figure 4C).

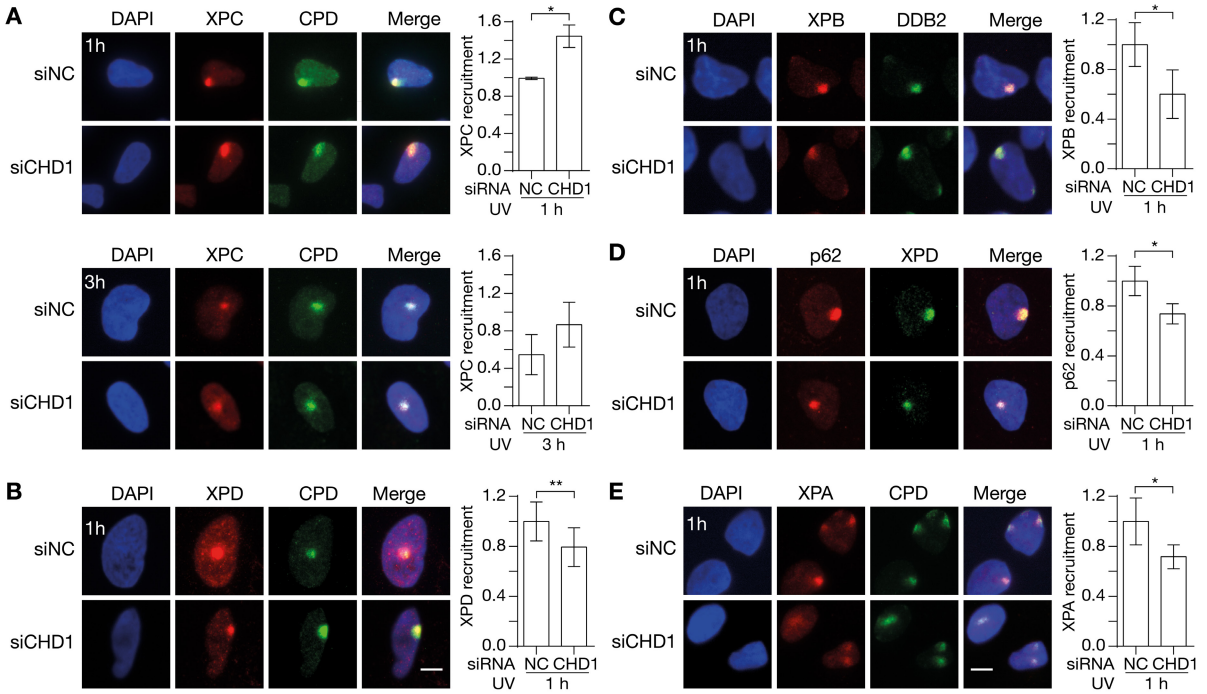

Figure 5 ***CHD1 promotes the transition from damage sensing to downstream GG-NER effectors****. (A) Increased accumulation of XPC protein. Representative immunofluorescence images of U2OS cells that were UV-irradiated through micropore filters to generate local spots of DNA damage. Immunostaining was carried out with antibodies against CPDs or XPC protein. Cells were pretreated with siRNA targeting the CHD1 transcript (siCHD1) or with non-coding control RNA (siNC). DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclear DNA. The recruitment of NER subunits was quantified by measuring spot intensities followed by normalization to the nuclear background (n = 6, 100 cells for each experiment). Scale bar: 10 μm. (B) Reduced recruitment to UV lesion spots of XPD, a subunit of the TFIIH complex, upon CHD1 depletion (n = 3, 100 cells for each experiment). (C) Reduced recruitment to UV lesion spots of XPB (another TFIIH subunit) upon CHD1 depletion (n = 3, 100 cells per experiment). DDB2 was used to demarcate the UV spots. (D) Reduced recruitment to UV lesion spots of p62 (yet another TFIIH subunit) upon CHD1 depletion (n = 6, 100 cells per experiment). XPD was used to demarcate the UV spots. (E) Reduced recruitment to UV lesion spots of XPA upon CHD1 depletion (n = 6, 100 cells per experiment).*
Data information: In A-E, data are presented as mean ± SEM. *P ≤ 0.05, **P < 0.01 (unpaired, two-tailed t-test).

In situ immunofluorescence is recognized as a straightforward tool to monitor GG-NER factors in living human cells (Volker *et al*, 2001; Fitch *et al*, 2003). This methodology was, therefore, used to examine the effect of CHD1 depletion on the recruitment of GG-NER factors to UV lesions. For that purpose, U2OS cells were irradiated with UV-C light through the 5-μm pores of filters to generate local spots of damage containing CPDs. Following 1 or 3 h of incubation, the formaldehyde-fixed cells were permeabilized and stained with antibodies against CPDs and different GG-NER proteins. These immunofluorescence studies revealed a differential effect of CHD1 depletion on distinct factors. Upon CHD1 down regulation, the level of the initial damage sensor XPC on spots of CPDs is increased relatively to controls (Figure 5A). This more abundant accumulation of XPC is observed at 1 h after UV irradiation. To quantify protein redistributions, the fluorescence intensity at damaged spots was divided by the background fluorescence measured in each nucleus. This procedure ensures that the

data demonstrate a truly increased accumulation of XPC protein at sites of damage rather than simply reflecting the higher overall level of this factor following CHD1 depletion. In contrast to this increased accumulation observed for XPC as the pathway initiator, the redistribution of downstream NER factors is reduced upon CHD1 depletion. A diminished recruitment to UV lesions following CHD1 depletion is observed for the TFIIH subunits XPD (Figure 5B), XPB (Figure 5C) or p62 (Figure 5D), and also for XPA protein (Figure 5E). This differential effect on factor recruitment to UV lesion sites indicates that CHD1 stimulates the transition during the GG-NER process from the XPC complex (the initial pathway initiator) to the follow-up factors TFIIH and XPA. As a consequence, in the absence of CHD1, XPC persists on UV lesion sites without being able to hand over the damage to downstream proteins in the GG-NER pathway.

CHD1 stimulates CPD excision and reduces UV cytotoxicity

The functional consequences of a CHD1 depletion were tested by monitoring the formation and excision of UV lesions in HeLa cells. The lack of CHD1 induced by siRNA treatment does not influence the MNase digestion pattern of chromatin (Figure 6A), indicating that the overall nucleosome assembly is unchanged. Consistent with this maintained chromatin configuration, the initial damage formation (frequency of CPDs and 6-4PPs) following UV irradiation is not affected by the lack of CHD1 (Figure 6B). However, the excision of CPDs is significantly slowed down upon CHD1 depletion. After 24 h of repair incubation, nearly 70% of the initial CPDs were excised in control cells but only 45-55% of CPDs were repaired in CHD1-depleted cells. This same inhibitory effect of CHD1 depletion was induced by three different siRNA sequences directed against the CHD1 transcript (Figure 6C). No inhibition by CHD1 depletion was detected for the repair of 6-4PPs, which are removed from the genome with faster kinetics than CPDs (Figure 6D).

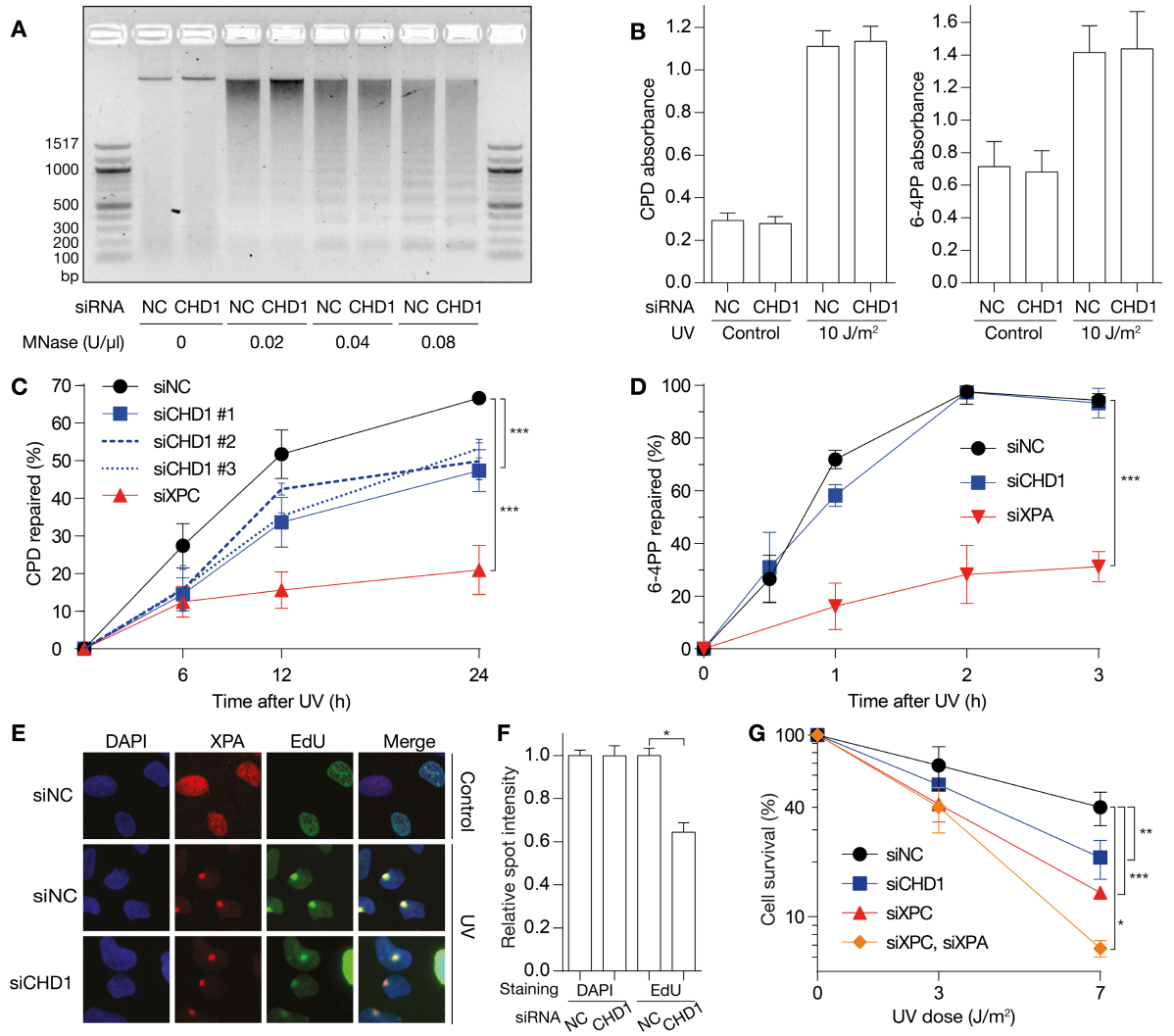

Figure 6 ***CHD1 stimulates CPD repair****. (A) MNase digestion of the chromatin of CHD1-depleted HeLa cells in comparison to control cells treated with non-coding RNA (siNC). Isolated chromatin was incubated with the indicated nuclease concentrations. (B) Initial damage formation following UV irradiation. HeLa cells were transfected with siCHD1 or siNC. Immunoassay absorbance values, providing a measure of UV lesions, were not affected by CHD1 depletions (n = 6, each experiment with 4 replicates). (C) Excision of CPDs in HeLa cells treated with siRNA targeting CHD1 or XPC, compared to transfections with siNC (n = 6, each experiment with 4 replicates). (D) Excision of 6-4PPs upon treatment with siRNA targeting CHD1 or XPA, in comparison to siNC (n = 3, each experiment with 4 replicates). (E) HeLa cells pre-treated with siRNA were irradiated through micropore filters. After 1 h of incubation with EdU, DNA repair spots were identified by staining with antibodies against XPA. EdU incorporation into DNA was detected by copper-mediated reaction with the Alexa 488 fluorophore. DAPI was used to stain the overall nuclear DNA. (F) DNA content (DAPI fluorescence) and unscheduled DNA synthesis (EdU incorporation) in CHD1-depleted cells normalized to control cells. S-phase cells were excluded from quantifications (n = 3, 100 cells per experiment). (G) HeLa cells transfected with the indicated siRNA sequences were UV-irradiated or mock-treated. Colony survival was quantified 7 days later and expressed as the percentage of controls (n = 3, each experiment with four replicates).*
*Data information: In B, C, D, F, G, data are presented as mean ± SEM. *P ≤ 0.05, **P < 0.01, ***P < 0.001 (unpaired, two-tailed t-test).*

To confirm the role of CHD1 in stimulating CPD excision, we compared the rates of DNA repair patch synthesis. For that purpose, spots of UV damage were generated in the nuclei of HeLa cells and unscheduled DNA synthesis in these spots was monitored for 1 h by measuring the incorporation of 5-ethynyl-2′-deoxyuridine (EdU). Figure 6E shows that the

reduced rate of CPD excision, detected upon CHD1 depletion, translates to lower levels of repair patch synthesis. The quantification of EdU signals at spots of DNA damage showed that DNA repair synthesis indeed takes place in CHD1-depleted cells at a significantly lower rate than in control cells (Figure 6F). Thus, CPD excision and unscheduled DNA synthesis are both significantly lower in cells depleted of CHD1 compared to controls. HeLa cell colony assays demonstrate that the reduced rate of CPD repair observed upon CHD1 depletion correlates with lower cell survival following UV irradiation (Figure 6G).

3.3.4 Discussion

The GG-NER system faces the challenge that it needs to process bulky base lesions in condensed chromatin, where the genomic DNA is organized in nucleosomes thought to act as physical barriers to damage recognition and repair (Thoma, 2005; Bell *et al*, 2011; Rodriguez *et al*, 2015; Dabin *et al*, 2016). Being the fundamental repeat unit of chromatin, each nucleosome consists of a core particle containing 147 base pairs of DNA wrapped around an octamer of two each of the basic histones H2A, H2B, H3 and H4. These nucleosome cores are spaced by linker DNA segments of variable lengths (generally 10-70 base pairs) and further associations with histone H1 promote higher levels of chromatin condensation (Grigoryev, 2012; Hergeth & Schneider, 2015). Within the 147 base pairs of nucleosome core DNA, CPDs arise with a distinctive periodicity pattern of 10.3-nucleotide intervals and are preferentially introduced at sites farthest from the surface of the histone octamer (Gale *et al*, 1987). Despite the conception that nucleosomes represent an accessibility barrier, GG-NER activity is rather efficient in processing CPDs throughout the human genome (see Figure 6C). The modulation of nucleosome dynamics by ATP-dependent chromatin remodelers has been identified as one important mechanism that promotes accessibility of the GG-NER complex to damaged DNA in eukaryotic cells (Ura *et al*, 2001; Gong *et al*, 2006; Klochendler-Yeivin *et al*, 2006; Ray *et al*, 2009; Zhang *et al*, 2009; Zhao *et al*, 2009; Jiang *et al*, 2010; Pines *et al*, 2012).

The present study was triggered by the role of transcription factors in DNA repair and the fact that at least one key element of the transcription machinery, the TFIIH complex, is engaged with initiation of transcription as well as initiation of NER activity (Compe & Egly, 2016). The chromatin remodeler CHD1 is yet another transcription factor that, like TFIIH, associates with the DNA template just downstream of the transcription start site during an early stage of transcription. CHD1 is recruited to the promoter-proximal nucleosomes of active genes and is thought to evict nucleosome cores to allow for promoter escape by RNA polymerase II. In the absence of CHD1 activity, RNA polymerase II remains sequestered on

the promoter-proximal nucleosomes immediately after the start of transcription (Skene *et al*, 2014). Another feature shared with the GG-NER process is the genome-wide range of CHD1 activity for example by driving stem cell pluripotency and transcriptional reprogramming (Park *et al*, 2014; Nodelman *et al*, 2016; Gaspar-Maia *et al*, 2009; Piatti *et al*, 2015). These considerations prompted us to test whether CHD1 may also have a chromatin remodeling function during early steps of the GG-NER pathway. We discovered (i) that CHD1 co-localizes with GG-NER factors in the chromatin of UV-irradiated cells (Figure 1), (ii) that the recruitment of CHD1 to UV damage occurs on nucleosome cores (Figure 2), (iii) that this UV-dependent CHD1 recruitment to chromatin relies on XPC protein (Figure 3), (iv) that CHD1 promotes the displacement of XPC from damaged sites, thus stimulating the recruitment of downstream GG-NER factors after initial lesion recognition (Figure 5) and, consequently, (v) that the lack of CHD1 slows down the excision of CPDs (Figure 6C) and enhances the cytotoxicity of UV light (Figure 6G). The effect of a CHD1 depletion on the repair of CPDs formed everywhere in the genome but not on the excision of 6-4PPs, formed predominantly in linker segments of euchromatin (Han *et al*, 2016; Mitchell *et al*, 1990; Lukáč *et al*, 1990) supports the notion that chromatin remodeling by CHD1 is required for ensuring the access of repair proteins to DNA damage located within nucleosome cores.

Although CHD1 co-localizes in chromatin with DDB2 (Figure 1), it might be surprising that its recruitment to nucleosomes depends on XPC protein but not on the accessory DDB2 subunit (Figure 3). Indeed, previous biochemical studies indicated that XPC protein loses the ability to interact with DNA once the substrate is wrapped around histone octamers in nucleosomes (Yasuda *et al*, 2005). On the contrary, reconstitution assays demonstrated that DDB2 is able to detect UV lesions even within nucleosome cores, suggesting that preceding DDB2-induced rearrangements of the nucleosome are indispensable before the DNA substrate becomes accessible to the XPC complex (Scrima *et al*, 2008; Fischer *et al*, 2011; Osakabe *et al*, 2015). It is important in this regard to point out that CPDs rather than 6-4PPs are the predominant UV lesions within nucleosome cores (Mitchell *et al*, 1990; Gale & Smerdon, 1990; Han *et al*, 2016) and that, when tested in binding assays with naked DNA substrates, the XPC complex is unable to sense the presence of CPDs (Sugasawa, 2001; Hey *et al*, 2002). If not embedded in chromatin, CPDs appear as non-distorting lesions that preserve Watson-Crick hydrogen bonding (Kim *et al*, 1995; Jing *et al*, 1998; McAteer *et al*, 1998) and are, therefore, invisible to the DNA damage sensing domains of XPC protein. However, a recent crystal structure of nucleosome cores containing CPD lesions revealed that, unlike their configuration in naked DNA, the two affected pyrimidines do not form proper Watson-Crick hydrogen bonds with the opposite purines and that these hydrogen bonds are actually destabilized at one pyrimidine of the CPD lesion (Horikoshi *et al*, 2016). This

substantial local distortion and base pair destabilization detected for CPDs located on nucleosome cores may render the lesion more conducive to recognition by the XPC subunit, such that at least a subset of CPDs in the nucleosome landscape of chromatin become amenable to the GG-NER process even in the complete absence of DDB2 protein. This direct recognition mechanism would explain why a considerable fraction of CPDs (~ 25% of total CPDs within 24 h) is still excised in *XP-E* cells in the absence of DDB2 activity (Hwang *et al*, 1999). Also, a local distortion of the CPDs induced by wrapping around histone octamers may account for the observation of Figure 3 that the XPC-dependent recruitment of CHD1 to nucleosomes can occur independently of any assistance by the DDB2 subunit.

To summarize, in addition to the previously reported DDB2-dependent remodeling mechanisms (Zhang *et al*, 2009; Zhao *et al*, 2009; Jiang *et al*, 2010; Pines *et al*, 2012), we identified CHD1 as a DDB2-independent remodeler facilitating GG-NER activity in chromatin. Our findings are consistent with a model by which CHD1 is required immediately after the initial recognition of CPDs within nucleosome cores to expel the histone octamer, which is a prerequisite for the recruitment of downstream factors like TFIIH and XPA. We further provide evidence that the function of CHD1 is not limited to nucleosome displacement. Indeed, in the absence of CHD1 activity, XPC accumulates on damaged sites indicating that CHD1 is not only required for the displacement of nucleosomes, but also for the concomitant removal of XPC interacting with nucleosomes. Collectively, these findings point to a mechanism by which CHD1 promotes the simultaneous displacement of both the histone octamer and XPC bound to damaged DNA wrapped around these histones. This unexpected scenario indicates that nucleosomes are not simply a physical barrier that impedes the access of repair factors to DNA but act as a structural scaffold that, in the presence of CHD1, facilitates the recognition and excision of a subset of CPD lesions.

3.3.5 Material and Methods

Cell lines. HeLa and HEK293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (low-glucose DMEM; ThermoFisher), U2OS cells (ATCC, cell type certified by STR profiling) in high-glucose DMEM (Sigma) supplemented with 10% (vol/vol) fetal calf serum (FCS, Gibco), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin.

RNA transfections. The following RNAi sequences were used: CHD1#1 (5'-CAUCAAGCCUCAUCUAAUAtt-3') from Ambion; CHD1#2 and CHD1#3 (5'-AUGCAGAAAUUAGGCGGUUUAtt-3' and 5'-AAGAUUCCGAUGACUCAUCAAtt-3') from Qiagen (CHD1 sequences #1 is used unless otherwise stated); DDB2 (5'-AGGGAUCAAGCAGUUAUUUGA-3') from Qiagen; XPA (5'-GCUACUGGAGGCAUGGCUAtt-3') from Qiagen and XPC (5'-TAGCAAATGGCTTCTATCGAA-3') from Microsynth. The non-coding control RNA was from Qiagen. Cells were transfected in 10-cm dishes with siRNA (10 or 16 nM) following the manufacturer's protocol for the Lipofectamine RNAiMAX (Invitrogen) reagent, and allowed to incubate for 48 h before starting the experiments.

Expression of mRNA. For gene expression analysis, total RNA from U2OS cells was isolated using an RNase isolation kit (Qiagen) according to the manufacturer's protocol. DNA was removed by DNase I (Qiagen) digestion. RNA concentration was determined in a NanoDrop instrument (Thermo Scientific). One µg RNA from each sample and 3 µg/µl random primers (Invitrogen) were subjected to reverse transcription (Roche) according to manufacturer's protocol. Fifty ng cDNA, 0.5 µl of FAM-tagged XPC primers (Life Technology) and 0.5 µl of VIC-tagged GAPDH primers (Life Technology) were applied to qRT-PCR according to manufacturer's protocol (Life Technology, TaqMan Fast Universal PCR Master Mix). The relative gene expression levels are presented as $2^{-\Delta\Delta CT}$ and normalized to the sample treated with noncoding siRNA.

Antibodies. The following antibodies, listed according to supplier, were used at the indicated dilutions. Abcam: mouse anti-DDB2 (ab51017, 1:50 for immunofluorescence, 1:200 for immunoblotting), mouse anti-XPC (ab6264, 1:1,000 for immunoblotting), mouse anti-p62 (ab55199, 1:300 for immunofluorescence). Cell Signaling: rabbit anti-caspase 3 (9501S, 1:1,000 for immunoblotting). Cosmo Bio: mouse anti-CPD [NMDND001, 1:1,000 for immunofluorescence, 1:5,000 for enzyme-linked immunosorbent assay (ELISA)], mouse anti-6-4PP (NMDND002, 1:1,000 for ELISA). Invitrogen: Alexa Fluor 488 and 594 goat anti-mouse IgG (1:400 for immunofluorescence). Protein-Tech: rabbit anti-CHD1 (20576-1-AP, 1:100 for immunoblotting). Santa-Cruz: mouse anti-CHD1 (sc-271626, 1:500 for immunoblotting), goat anti-H3 (sc-8654, 1:10'000 for immunoblotting), rabbit anti-XPB (sc-293, 1:100 for immunofluorescence), rabbit anti-XPA (sc-853, 1:100 for immunoblotting). Sigma: mouse

anti-α-tubulin (T5168, 1:10,000 for immunoblotting), mouse anti-FLAG M2 (F3165, 1:1,000 for immunoprecipitation), rabbit anti-XPC (X1129, 1:100 for immunofluorescence), peroxidase anti-mouse IgG (1:20,000), peroxidase anti-rabbit IgG (1:20,000).

UV irradiation. Exposure to UV-C light was carried out in culture dishes at the indicated doses with a germicidal lamp (wavelength 254 nm) after washing the cells with phosphate-buffered saline (PBS) and removal of residual buffer. Local damage was generated by irradiation with 100 J/m^2 through a 5-µm polycarbonate filter (Whatman). After UV irradiation, the cells were incubated for the indicated times with fresh culture medium.

Immunoblotting. Cells were treated as indicated, washed with Puck's EDTA (137.0 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 4.2 mM $NaHCO_3$, 0.7 mM EDTA) and lysed in 100 µl of 1% Triton buffer [150 mM KCl, 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5% (vol/vol) glycerol, 1 mM N-ethylmaleimide and 1% (vol/vol) Triton X-100] supplemented with protease inhibitor cocktail (Roche). Protein concentrations were measured by the bicinchoninic acid assay (Pierce). A Laemmli buffer stock [final concentration: 63 mM Tris-HCL, pH 6.8, 10% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 0.0005% (wt/vol) bromophenol blue] was added and the samples were heated to 95°C for 5 min. Fifty µg of sample proteins were separated in 4-20% Criterion TGX Stain-Free precast gels (BioRad) for 22 min at 300 V and transferred to PVDF membranes using the Turbo transfer device (BioRad, 7 min at 5 A).

Chromatin digestion. Chromatin was fragmented as described (Fei *et al*, 2011). Protein synthesis was inhibited by the addition of cycloheximide (100 µg ml^{-1}; Sigma) for 30 min prior to UV irradiation. Cells were irradiated with the indicated UV-C doses and lysed on ice with NP-40 buffer [25 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) NP-40, 0.25 mM phenylmethylsulfonyl fluoride and EDTA-free protease inhibitor cocktail (Roche)] (Sugasawa *et al*, 2005). Lysis was carried out for 30 min on a turning wheel. Free proteins not bound to chromatin were recovered in the supernatant after centrifugation (10 min at 15'000 g). The remaining pellet was resuspended in CS buffer [20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose and 0.1 % (vol/vol) Triton X-100] (Kapetanaki *et al*, 2006). This mixture was supplemented with 10-fold reaction buffer [500 mM Tris-HCl, pH 7.9, 50 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin (New England Biolabs). MNase (4 U/µl; New England Biolabs) was added and digestion carried out for 20 min at 37°C. The solubilized constituents were separated from the insoluble pellet by centrifugation (10 min, 15'000 g) after adding EDTA (5 mM final concentration) to stop the reaction.

MNase profiling. U2OS cells were irradiated with UV-C (10 J/m^2) and lysed on ice 1 hour later with NP-40 buffer, the remaining pellet was resuspended in CS buffer and the mixture

was supplemented with 10-fold reaction buffer as outlined above. MNase was added in different concentrations, the digestion carried out for 5 min at 37°C and the reaction stopped with 5 mM EDTA. The DNA was extracted from each digested sample (50 µl) by adding 150 µl TE buffer [10 mM Tris-HCl, pH 8.0, 1 mM EDTA] and 200 µl neutral phenol (ThermoFisher). After shaking for 15 min and centrifugation (5 min at 6,000 g) the phenol was discarded and the aqueous solution was washed twice with 200 µl chloroform. The DNA was precipitated with ethanol in the presence of 100 mM sodium acetate, dried and resuspended in TE buffer. DNA concentrations were determined in the NanoDrop device.

Pull-down of chromatin-associated GG-NER complexes. HEK293 cells were transiently transfected at 80% confluency with plasmid DDB2-p3XFLAG-14-N3 (10 µg) according to the manufacturer's protocol for the FuGENE® HD reagent (Roche), and UV-irradiated 24 h later. Following another 30 min, the cells were lysed on ice in NP-40 buffer, the remaining pellet was resuspended in CS buffer and the mixture was supplemented with 10-fold reaction buffer. The MNase digestion (4 U/µl) was carried out for 20 min at 37°C. The residual insoluble chromatin was recovered by centrifugation (10 min, 16'000 g) and resuspended by sonication on an ice-water bath (3 cycles of 30 s with 30-s intervals) in CS buffer. Subsequently, nucleoprotein complexes bound to the DDB2-FLAG prey were purified using 40 µl of anti-FLAG M2 Affinity Gel (Sigma) in the presence of IP buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% (vol/vol) NP-40, 2 mM β-mercaptoethanol, 5% (vol/vol) glycerol and all protease inhibitors]. Beads were incubated overnight at 4 °C with the sonicated mixture, washed once with TNET [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (vol/vol) Triton X-100 and protease inhibitors] and once with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and protease inhibitors). The beads were heated to 95°C for 5 min in 10 µl TBS complemented with 5.0 µl of Laemmli buffer stock. The samples were separated on a 10% (wt/vol) denaturing polyacrylamide gel and transferred to PVDF membranes. Proteins were subsequently detected by immunoblotting.

Pull down of histones. After washing on ice with PBS, 10 µl of slurry Protein G sepharose (GE Healthcare) were incubated for 45 min at 4°C on a turning wheel with H3K4me3 antibodies (Abcam, ChiP Grade ab8580, 2 µg of antibodies for 25 µg of chromatin proteins). After centrifugation (1 min, 100 g), protein G-sepharose was suspended in buffer A [0.5 M Tris-HCl, pH 8.0, 20% (vol/vol) glycerol, 300 mM NaCl, 2% (vol/vol) Triton X-100, 2 mM EDTA, 0,25 mM phenylmethylsulfonyl fluoride and ETDA-free protease inhibitor cocktail (Roche)], added to fragmented chromatin and incubated for 3 h at 4°C on a turning wheel. The beads were washed twice by centrifugation (2 min, 100 g) in HNTG buffer [20 mM HEPES, pH 7.5,150 mM NaCl, 0.1% (vol/vol) Triton X-100 and 10% (vol/vol) glycerol] and the samples were analysed by polyacrylamide gel electrophoresis and immunoblotting.

Immunofluorescence microscopy. Cells were grown on 12-mm glass coverslips (Thermo Scientific) to 80% confluency and irradiated through filters to induce local spots of UV damage. After the indicated repair times, cells were processed with pre-extraction buffer [25 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% (vol/vol) Triton X-100] added for 2.5 min at 4°C. Next, cells were fixed with 4% (wt/vol) paraformaldehyde for 15 min and permeabilized for 20 min with PBS containing 0.1% (vol/vol) Tween 20. Following a 30-min blocking step with PBS containing 20% (vol/vol) FCS, primary antibodies were diluted in PBS containing 5% (vol/vol) FCS and applied for 1 h at 37°C. Washing with PBS-0.1% Tween 20 was followed by incubation with secondary antibodies and DAPI (0.2 µg ml^{-1}), diluted in PBS containing 5% FCS, for 1 h at 37°C. Images were taken with a bright field microscope (Leica, 63x oil Plan-Apochromat, 1.4 numerical aperture oil immersion lens) and analysed using the Image J software. The fluorescence of 100 nuclei was examined and the accumulation of proteins at UV lesion sites is expressed as the ratio of fluorescence signal intensity in damaged spots relative to the signal intensity of the surrounding nuclear area.

Unscheduled DNA synthesis. The synthesis of DNA repair patches was measured by a fluorescence-based method (Nakazawa *et al*, 2010). HeLa cells were seeded on 12-mm coverslips and locally UV-irradiated. Subsequently, the culture medium was supplemented with 10 mM 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) followed by another 1 h of incubation. Cells were washed with PBS, preextracted for 2.5 min, fixed with 4% (wt/vol) paraformaldehyde for 15 min and permeabilized for 20 min. Antibodies against XPA protein were applied as described above. Incorporated EdU was coupled to Alexa Fluor 488 using the Click-it kit as instructed by the manufacturer (Invitrogen). Images were obtained by microscopy with the Leica instrument and analysed using the Image J software. For quantifications, EdU incorporation was measured in 100 cells by determining fluorescence intensity in the UV-damaged areas (marked by XPA staining) divided by the background nuclear intensity. S-phase cells displaying high EdU fluorescence across their entire nucleus were excluded.

Quantification of UV lesions. Formation and removal of UV lesions was detected by ELISA as described (Fei *et al*, 2011). Briefly, whole-genome DNA was extracted using the DNeasy kit (Qiagen) and denatured by heating to 99ºC for 10 min, followed by a 15-min incubation on ice. A volume of 50 µl per well of denatured DNA (at a concentration of 4 µg/ml for 6-4PP detection, 200 ng/ml for CPD detection) was distributed into a 96-well microtiter plate (Greiner) coated with protamine sulfate (Sigma) and dried overnight at 37ºC. The DNA-coated plates were washed five times with PBST [0.05% (vol/vol) Tween-20 in PBS] and blocked with 2% (vol/vol) FBS in PBS at 37 ºC for 60 min. The antibodies against either 6-4PPs (64M-2) or

CPDs (TDM-2) were applied for 30 min (37 °C). Primary antibodies bound to DNA were recognized by biotin-labelled $F(ab')_2$ fragments of anti-mouse IgG (1:2,000; Invitrogen) added for 30 min at 37°C. After washing the plates, 100 µl of a peroxidase-streptavidin conjugate (1:10.000; Invitrogen) was distributed into each well. The reaction was started by adding 0.5 mg/ml o-phenylenediamine, 0.007% (vol/vol) H_2O_2 and citrate-phosphate buffer (50 mM Na_2HPO_4, 24 mM citric acid, pH 5.0), stopped with 50 µl of 2 M H_2SO_4, and monitored by measuring the absorbance at 492 nm in a PLUS384 microplate spectrophotometer (Molecular Devices).

Survival (colony forming) assay. HeLa cells treated as indicated were seeded in different dilutions and left for 7 days at 37°C to allow for colony formation. The growing colonies were stained with 0.5% (w/v) crystal violet in 80% ethanol and counted.

Cell cycle analysis. HeLa cells were arrested in G1 by a 24-h treatment with mimosine (0.5 mM, Sigma) (Galgano & Schildkraut, 2006). UV-exposed cells were allowed to recover for the indicated times and labelled with 10 µM EdU for 1 h prior to harvesting. Cells cycle profiles were analysed using the Life Technologies Click-iT Edu Alexa Fluor 488 Flow Cytometry Assay kit. Briefly, cells were fixed in 1% (wt/vol) PFA/PBS (Sigma) for 10 min and permeabilized in saponin buffer for 10 min; 200,000 cells were incubated with a mouse anti-γH2AX antibody (Millipore, 1:2000) for 1.5 h and with an Alexa Fluor 647 anti-mouse antibody (Invitrogen A31571, 1:50) for 30 min. EdU was coupled to Alexa Fluor 488 azide for 30 min. Cells were treated with 0.1 mg/ml RNase and DNA was stained with 1 µg/ml DAPI, followed by analysis in a CyAn ADP flow cytometer (Beckman Coulter). Results were analysed with Flow Jo 10 data analysis software (FLOWJO, LLC).

Statistics. GraphPad Prism 6 was used to perform unpaired, two-tailed *t*-tests as indicated in the figure legends. One-sample t test with a hypothetical value of 1.0 was applied for independent immunoblot assays. *P* values expressed as $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to indicate statistical significance.

3.3.6 Acknowledgements

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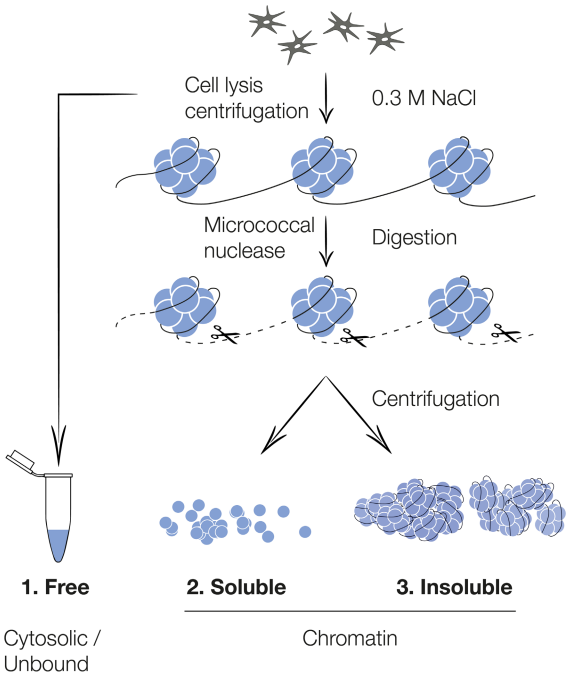
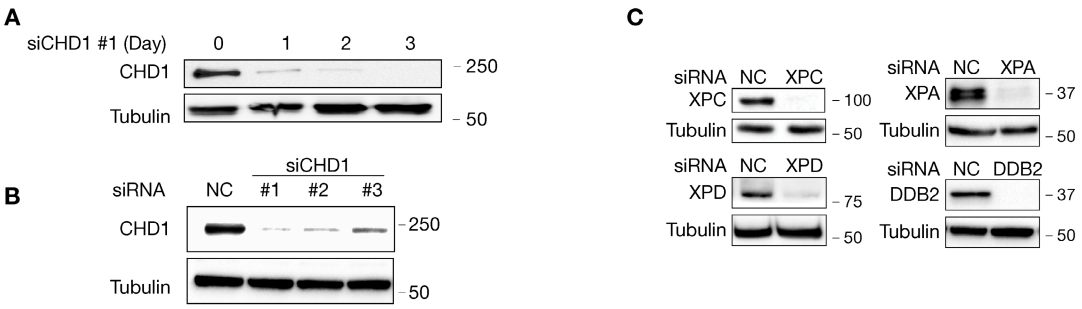
3.3.7 Author contributions

P.R. and H.N. devised and planned the experiments, P.R., Z.G. and C.B.P. carried out the experiments and analyzed the data, P.R. and H.N. wrote the manuscript.

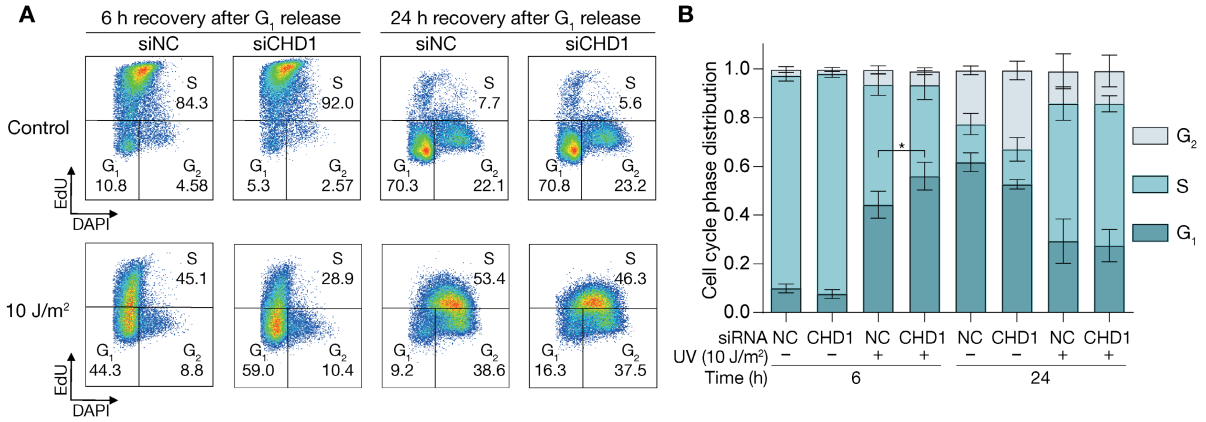
3.3.8 Supplementary Figures



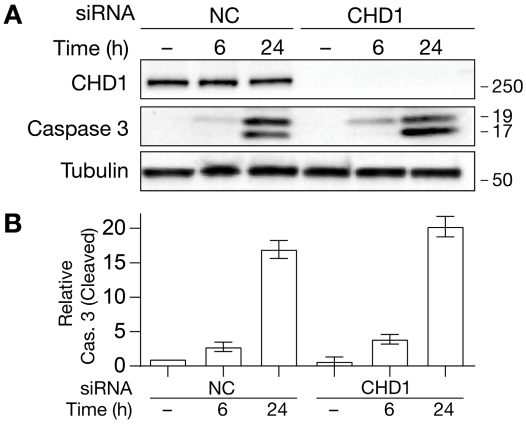
Supplementary Figure 1 ***Chromatin fractionation.*** *Flow diagram illustrating the chromatin dissection after removal of unbound or loosely bound (free) proteins by salt extraction (0.3 M NaCl). MNase digestion generates a supernatant of solubilized chromatin proteins and a remaining insoluble pellet of condensed nucleosome cores.*



Supplementary Figure 2 ***Efficiency of siRNA-mediated protein depletions.*** *(A) Immunoblots of whole cell lysates were carried out at different time points (1, 2 and 3 days) after transfection with an siRNA sequence (#1, 16 nM) directed against the CHD1 transcript. (B) Protein depletion achieved 2 days after transfection with alternative siRNA sequences (#2 and #3. 16 nM) directed against the CHD1 transcript. (C) Protein depletion achieved 2 days after transfection with the indicated siRNA sequences (16 nm). Tubulin served as the loading control.*



Supplementary Figure 3 ***Cell cycle arrest induced by UV irradiation.*** *(A) HeLa cells released from mimosine-induced G1 arrest 6 and 24 h before analysis. DNA replication was monitored by EdU incorporation. Cells were fixed, stained with DAPI and analyzed by flow cytometry. (B) Quantification of cell cycle phases. The increases degree of G1-S arrest, in cells depleted of CHD1 and subsequently UV-irradiated, is consistent with their reduced efficiency in repairing CPDs.*
*Data information: In B, data are presented as mean ± SEM (n = 3 independent experiments). *P ≤ 0.05 (unpaired, two-tailed t-test).*



Supplementary Figure 4 ***Apoptotic response.*** *(A) Caspase 3 cleavage indicating that the depletion of CHD1 does not induce apoptosis in unchallenged U2OS cells. Caspase 3 cleavage is observed upon UV irradiation (10 J/m^2), particularly following 24 h-long incubations. The cleavage products migrate as polypeptides of 17 and 19 kDa. Tubulin served as the loading control. (B) Quantification of immunoblots. The slightly increased caspase 3 activation observed when comparing UV-irradiated cells, previously transfected with non-coding RNA (NC) or siRNA against CHD1, is consistent with a role of CHD1 in the repair of UV lesions.*
Data information: In B, data are presented as mean ± SEM (n = 3 independent experiments).

4 Conclusion

In human cells, faithful repair of DNA damage induced by the UV spectrum of solar light, i.e., cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), requires a complex cascade of recognition and excision events. Since the discovery of DNA photolyases and of "Dark Repair" by Prof. Aziz Sancar 38 and 33 years ago (Sancar & Rupert, 1978; Sancar & Rupp, 1983), immense progress has been made in the understanding of how UV damage in DNA is processed . The dogma of a stable and immutable DNA molecule was abandoned in favour of a concept of perpetual DNA damage formation by exogenous and endogenous influences and subsequent DNA restoration by repair. It came to light that *three* entirely different UV damage repair pathways evolved in different species: (*i*) Direct repair by photolyases in many organisms but not placental mammals, (*ii*) Global-genome nucleotide excision repair (NER) as the UvrABC system in prokaryotes *or* the multiprotein NER system in eukaryotes *and* (*iii*) Transcription-coupled NER in both prokaryotic and eukaryotic cells.

With the increasing genome size in higher eukaryotes and the need for a more efficient DNA storage system, chromatin increased in complexity. As a consequence, the DNA accessibility for DNA repair proteins decreased, thus challenging repair efficiency. In particular, the multiprotein global-genome NER system was compelled to cope with different compaction states of chromatin to overcome this accessibility problem. A possible solution might be that NER proteins recruit enzyme complexes that are capable of modelling the chromatin structure to gain sufficient accessibility to the DNA substrate. Based on this hypothesis, this thesis was aimed at the characterization of possible interplays between the NER system and ATP-dependent enzyme complexes that are known to regulate dynamically DNA and chromatin configurations.

In a first study (in collaboration with *Nadine Mathieu), we* demonstrate that the XPD helicase, one of the transcription factor IIH (TFIIH) subunits, plays an important role in damage verification and demarcation of the lesion site in chromatin. Two newly engineered XPD mutants (Y192A and R196E) were unable to sequester the UV lesion in the DNA double helix and, therefore, unable to trigger DNA repair activity (Mathieu *et al*, 2010). The UV-dependent sequestration of XPD in chromatin was abolished despite the fact that these mutants still assemble into the TFIIH complex, remain transcription-proficient and preserve DNA helicase activity. These findings indicate that the XPD subunit not only works as a helicase that unwinds the double helix but also as DNA scanning enzyme and ultimate damage recognition factor downstream of the initial damage sensors UV-DDB and XPC (in the global-genome NER pathway) or the stalled RNA polymerase (in the transcription-coupled NER pathway).

This bipartite recognition step, involving UV-DDB/XPC and XPD, explains how the global-genome NER system is able to process a wide diversity of DNA lesions.

In a second report (in collaboration with *Marjo-Riita Puumalainen),* we show that a tight spatiotemporal regulation of XPC and DDB2 proteins is crucial for proficient global-genome NER activity (Puumalainen *et al*, 2014). DDB2 is the DNA-binding subunit of the dimeric UV-DDB complex. An abnormal retention of XPC and stabilisation of DDB2 at the lesion sites causes decreased NER efficiency and genome instability. The ubiquitin-dependent p97/VCP segregase complex is responsible for targeting and removing the global-genome NER damage sensors (XPC and DDB2) directly from the chromatin after their binding to the UV lesions. These findings indicate that not only the entry of NER factors into chromatin is indispensable for successful repair but also their exit from chromatin after completion of their function in recognizing DNA damage and triggering the downstream reactions.

The central question of my project was to investigate whether chromatin remodelling, besides being fundamental for transcription or replication, serves as a toolkit to facilitate DNA repair, specifically global-genome NER activity. At the outset of my study, several reports had already shown that subunits of ATP-dependent chromatin remodelling complexes (INO80, SNF2H, ALC1 and BRG1) support the NER reaction in eukaryotic cells (Gong *et al*, 2006; Klochendler-Yeivin *et al*, 2006; Ray *et al*, 2009; Zhang *et al*, 2009; Zhao *et al*, 2009; Jiang *et al*, 2010; Sarkar *et al*, 2010; Aydin *et al*, 2014a; Pines *et al*, 2012). These studies revealed an impaired CPD repair after depletion of the corresponding remodelling complex, but the molecular mechanisms by which such remodelling complexes interact with the NER system remained unknown. In an initial siRNA screen targeting multiple chromatin remodelling complexes from different subfamilies, I could confirm that the above mentioned remodelling subunits indeed stimulate the excision of CPDs but not 6-4PPs, but I also found a new candidate accessory factor for global-genome NER activity. This accessory factor consists of CHD1 (for chromodomain helicase DNA-binding 1), which is an ATPase able to displace nucleosome cores and thereby grant access to otherwise histone-blocked DNA sites. By immunoprecipitation of nucleoprotein complexes from the fragmented chromatin of human cells, I discovered that CHD1 co-localizes with global-genome NER factors in response to UV irradiation. Fractionation with micrococcal nuclease showed that CHD1 is recruited to the UV-damaged nucleosomes of condensed chromatin, consistent with a role in displacing these nucleosomes to allow for excision repair. Subsequent depletion studies using siRNA revealed that the UV-dependent relocation of CHD1 to nucleosomes depends on XPC protein (but not DDB2) as the initiating damage sensor. We conclude that XPC detects the damage and recruits CHD1 to the chromatin. Thus, against the general assumption that XPC is unable to access histone-bound DNA, associations of XPC with nucleosomes serve as an intermediate

damage recognition scaffold. The requirement for a chromatin remodeller to translate this recognition intermediate to downstream global-genome NER events is demonstrated by the finding that CHD1 stimulates CPD excision and protects from UV-induced cytotoxicity. By displacing nucleosomes at the lesion sites, CHD1 allows for the subsequent recruitment of the large multiprotein complex TFIIH which unwinds the DNA and verifies the lesion. In turn, TFIIH recruits further NER factors including the endonucleases XPF-ERCC1 and XPG, which together cleave the damaged DNA strand on either side of the lesion. After DNA repair patch synthesis and DNA ligation, the chromatin is restored as shown (Polo, 2015). A model summarizing these findings is illustrated in Figure 7.

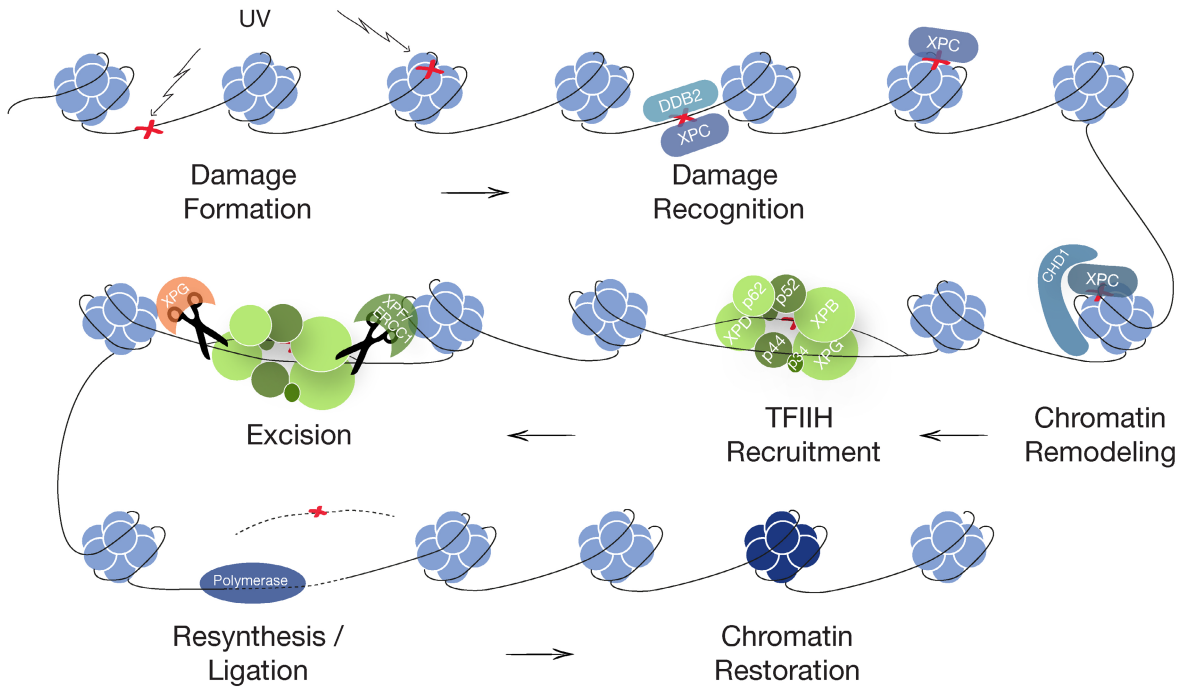


Figure 7 ***CHD1-dependent chromatin relaxation upon UV irradiation.*** *After induction of CPDs by UV light, the global-genome NER damage sensors DDB2 and XPC are recruited to UV lesions. DDB2 is required for the initial detection of CPDs on segments of naked DNA. Instead, the local base pair distortion induced by wrapping around histones allows XPC protein to detect CPDs directly using the nucleosomes as a recognition scaffold. The damage recognition complex containing XPC recruits CHD1, which displaces the nucleosome. The lesion is now accessible for assembly of the multiprotein TFIIH complex and lesion verification by XPD. The damaged DNA strand gets excised and helix integrity is restored by DNA resynthesis and DNA ligation. The repair process is completed by chromatin reassembly.*

We were surprised to see that the UV-dependent recruitment of CHD1 to nucleosomes depends on XPC protein but not the DDB2 subunit. On the one hand, previous *in vitro* biochemical studies indicated that XPC protein loses the ability to interact with DNA once the substrate is wrapped around histones in the nucleosomes (Yasuda *et al*, 2005). Instead, DDB2 retains the ability to detect UV lesions within nucleosome cores (Scrima *et al*, 2008; Fischer

et al, 2011; Yeh *et al*, 2012; Osakabe *et al*, 2015; Horikoshi *et al*, 2016), suggesting that DDB2-induced rearrangements of the nucleosome are indispensable before the DNA substrate becomes accessible for XPC complex. On the other hand, when tested with naked DNA substrates XPC protein is also unable to sense the presence of CPDs (Sugasawa, 2001; Hey *et al*, 2002) as XPC recognizes ruptured base pairs but CPDs preserve Watson-Crick hydrogen bonding between base pairs (Kim *et al*, 1995; Jing *et al*, 1998; McAteer *et al*, 1998). A crystal structure of nucleosome cores containing CPDs revealed, however, that unlike the native configuration found with naked DNA the two affected pyrimidines of the CPD do not form proper hydrogen bonds with the opposite purines, and that the Watson-Crick hydrogen bonds are destabilized at one of the affected pyrimidines of the CPD lesion (Horikoshi *et al*, 2016). This local base pair destabilization found for CPDs located on nucleosome cores may render the lesion more conducive to recognition by the XPC subunit, such that at least a subset of CPDs in the nucleosome landscape of chromatin become amenable to the global-genome process even in the absence of DDB2. This predicted mechanism would explain why a considerable fraction of CPDs (~ 25% of total CPDs within 24 h) is still excised in *XP-E* cells characterized by a complete lack of DDB2 activity or upon depletion of DDB2 (Hwang *et al*, 1999; Puumalainen *et al*, 2014). This local distortion of the CPDs induced by the wrapping around histones may also account for our observation that the XPC-dependent recruitment of CHD1 to nucleosomes occurs in the absence of DDB2.

In conclusion, these findings are consistent with a model of global-genome NER activity in chromatin by which CHD1 is required immediately after the initial recognition of CPDs located within nucleosomes to expel the histone octamer, which in turn allows for the recruitment of downstream factors in the pathway responsible for excision and DNA repair patch synthesis (Figure 1). Our findings also point to a mechanism by which CHD1 promotes the simultaneous removal of both nucleosome cores and XPC bound to damaged DNA wrapped around the histones. This mechanism indicates that the nucleosomes are not simply a physical barrier to DNA repair but act as a structural scaffold to facilitate the recognition of bulky lesions as well as the XPC-TFIIH transition in the global-genome NER pathway. Collectively, the presented results presented here illustrate that global-genome NER and probably DNA repair in general are tightly connected with chromatin dynamics.

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Education

2012 – 2016	Ph.D. in Cancer Biology, Institute of Veterinary Pharmacology and Toxicology, Dr. Prof. Hanspeter Nägeli, University of Zürich, Switzerland
	Employment as a PhD student at UZH since Feb. 2012
	Project title: «How Global-Genome Nucleotide Excision Repair Enters and Exits Chromatin»
2011	Scientific advisor at Streuli Pharma AG
2009 –2010	*Master of Science in Biochemistry (Chemistry/Biophysics Track)* at Faculty of Science (University of Zürich), Graduation: 07.03.2011 / 1.5 years
2010	*Master project* in the group of Prof. Dr. Josef Jiricny, Duration: 9 months
	Project title: «Studies on posttranslational protein modification in DNA mismatch repair»
2006 – 2009	*Bachelor of Science in Biochemistry (Chemistry track),* Graduation: 28.09.2009 / 3 years
2002 – 2006	*Matura in Biology and Chemistry* at Kantonsschule Wattwil SG, Graduation: March 2006, 4 years

List of Publications

Rüthemann, P., Balbo Pogliano, C. & Naegeli, H. Global-genome Nucleotide Excision Repair Controlled by Ubiquitin/Sumo Modifiers. *Front. Genet*. 7, 1–10 (2016).

Puumalainen, M. R**., Rüthemann, P**., Min, J. H. & Naegeli, H. Xeroderma pigmentosum group C sensor: Unprecedented recognition strategy and tight spatiotemporal regulation. *Cell. Mol. Life Sci.* 73, 547–566 (2016).

Puumalainen M-R, Lessel D, **Rüthemann P**, Kaczmarek N, Bachmann K, Ramadan K & Naegeli H. Chromatin retention of DNA damage sensors DDB2 and XPC through loss of p97 segregase causes genotoxicity. *Nat. Commun.* **5:** 3695 (2014).

Mathieu, N., Kaczmarek, N., **Rüthemann, P**., Luch, A. & Naegeli, H. DNA Quality Control by a Lesion Sensor Pocket of the Xeroderma Pigmentosum Group D Helicase Subunit of TFIIH. *Curr. Biol.* 23, 204–212 (2013).

Teaching and Mentoring Experiences

2012	Supervision: Introduction to cell biology and cell culture for veterinary students
2013	Supervision: Introduction to cell biology and cell culture for veterinary students
2014	Supervision of Master student and support of master thesis of Karin Bachmann
2016	Lecture (Cell biology, 2 hours) at ETH, Chemist master students

Attended Conferences and Meetings

March 29-31, 2016	6th Student Retreat of the Cancer Biology PhD Program, Davos (Switzerland), Talk
September 08-12, 2014	13th Biennial Meeting of the DGDR, Mainz (Germany), *oral presentation*
February 12-14, 2014	5th Student Retreat of the Cancer Biology PhD Program, Filzbach (Switzerland), *oral presentation*
October 7-10, 2013	German – French Repair Meeting on Epigenetics and Genome Integrity, Strasbourg – Illkirch (France), *oral presentation*
April 11-13, 2013	5th Cancer Network Zurich Retreat, Grindelwald (Switzerland), *poster presentation*

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